MICROBIOME DEPENDENT AND INDEPENDENT DETERMINANTS OF *PLASMODIUM* INFECTION IN THE MOSQUITO *ANOPHELES GAMBIAE*

by

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Abstract

The biology of *Anopheles* mosquitoes has been heavily investigated due to their ability to transmit *Plasmodium* parasites that cause malaria. In particular, interactions between the mosquito immune system, *Plasmodium* parasites, and the endogenous midgut microbiota are critical determinants of the outcome of pathogen infection and transmission. In this regard, we have investigated bacteria-independent, *Plasmodium*-specific mosquito immune responses as well as the role fungi play in mosquito-pathogen interactions; two areas of research that have not been well studied. Using whole-genome transcript microarray analysis, RNAi-mediated gene silencing, *Plasmodium* infection assays, and other tools we reported two novel findings. First, we identified the existence of bacteria- and IMD pathway-independent anti-*Plasmodium* defenses. This finding highlights non-canonical anti-*Plasmodium* defenses that could be exploited to interrupt pathogen transmission. Second, we showed that the non-pathogenic fungus *P. chrysogenum* is capable of enhancing *Plasmodium* susceptibility in *Anopheles* mosquitoes. To our knowledge, this is the first report of a fungus capable of increasing *Plasmodium* infection, which has implications for the success of *Plasmodium* transmission in nature. Overall, we elucidated a novel immune

mechanism as well as a new microbial component influencing mosquito-pathogen interactions.

Keywords: *Anopheles*, immunity, *Plasmodium*, bacteria, fungi, microbiome

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Alternates: Dr. Douglas Norris and Dr. Michael Matunis

Preface

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Chapter 1: Introduction

1.1 Human malaria is a global peril

Human malaria is a persistent public health threat of global prominence. In 2012, there were an estimated 207 million cases and 627,000 deaths attributed to malaria (WHO 2012). Worldwide, human malaria infection is the 8th leading cause of Disability Adjusted Life Years (DALYs) and is the 2nd leading cause of DALYs in Africa (Snow et al. 2003). Ongoing parasitic infections of humans have resulted in enormous monetary losses due to immeasurable lost productivity and economic development (WHO 2008). Over the years, billions of dollars have been invested in malaria intervention strategies. Historically, humans have heavily relied on anti-malarial drugs and insecticides to control parasites and mosquito vectors (Prevention 2014a). However, human malaria infections continue to occur due to insecticide resistant vectors, drug resistant parasites, compliance failures, poverty, non-cooperative target populations, and other challenges (WHO 2008). These failures have stipulated the need for integrative malaria interventions that utilize novel scientific research and socially acceptable

strategies to interrupt transmission at all stages of the parasite life cycle (WHO 2008).

1.2 Vector-based malaria interventions

Human malaria parasites are single-celled protozoan parasites belonging to the genus *Plasmodium*. These parasites have distinct human and insect components during their complex life cycle (Prevention 2014b). For an overview of the malaria life cycle, see Figure 1.1. Human *Plasmodium* parasites have exploited the anautogeny of female mosquitoes in order to be transmitted from an infected to susceptible human host. These human pathogens infect and are exclusively vectored by mosquitoes in the genus *Anopheles* (Prevention 2014b). Therefore, *Anopheles*-based transmission is essential for parasite survival. This weakness in the parasite life cycle can be attacked by targeting the *Plasmodium* parasites within the mosquito or the mosquito vectors themselves. These strategies are referred to as vector-based malaria interventions.

Past vector-based malaria interventions have been successful in portions of Europe and North America (Prevention 2014a). Human malaria has been eliminated from these regions due in principle to the control of

mosquito vector populations. Traditional interventions include the destruction of mosquito breeding sites and the use of insecticidal agents. The mosquito vectors still persist in these locations, and imported human malaria cases from endemic regions occasionally results in local transmission (Filler et al. 2006). A multitude of factors including high socioeconomic status, rapidly available drug treatment, and developed infrastructure contributes to these regions remaining malaria free. Conversely, endemic malaria can be found in Central America, South America, Southeast Asia, and most importantly of all Africa where traditional control measures have failed. Drug resistant parasites, insecticide resistance, inaccessible breeding sites,

Figure 1.1: The Plasmodium Life Cycle

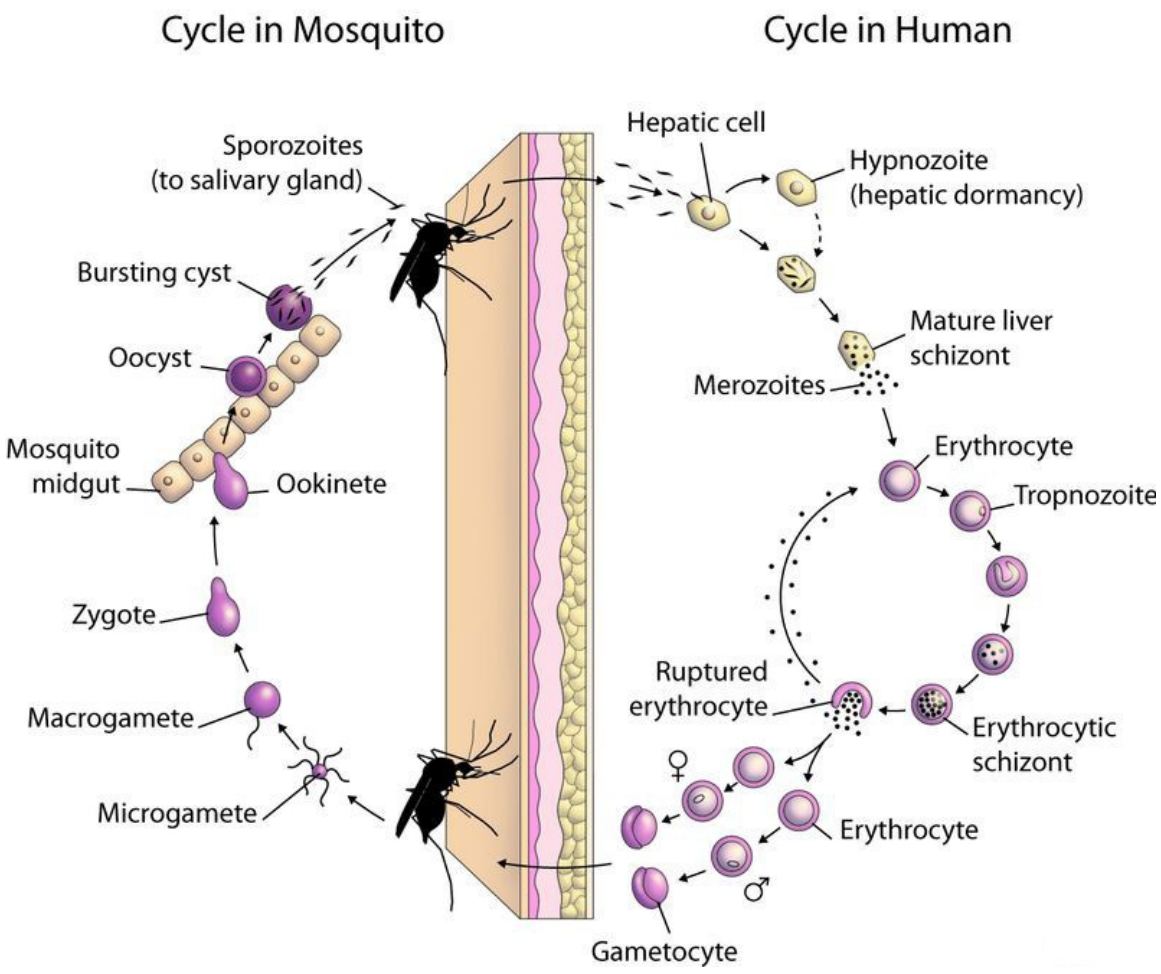

Figure 1.1: Life cycle of the malaria parasite. Adapted from http://ocw.jhsph.edu (2011). The left side of the figure depicts the stages in the mosquito, whereas the right side of the figure depicts the stages in humans. Although the timing of each stage may vary between *Plasmodium* species, the overall life cycle is similar. For a detailed description of the life cycle, see Section 1.4.

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underdeveloped infrastructure, and compliance issues are only some of the causes of continued malaria endemnicity in these regions. The failure of these traditional control strategies has driven the demand for new vector-based

malaria interventions. For a review of vector-based malaria interventions, see Figure 1.2 (José L Ramirez, Garver, and Dimopoulos 2009).

Anopheles gambiae is the primary vector of *Plasmodium falciparum* malaria in Africa, and one of the most important vectors worldwide (White, Collins, and Besansky 2011). The survival of *Plasmodium* is completely dependent on the mosquito vector, and this vulnerability of the parasite has been a driving force behind investigations into tripartite interactions between the mosquito, the parasite, and the environments they reside in. Over the years, a large amount of research has characterized *A. gambiae* biology and provided tools for vector-based interventions. Traditional vector ecology studies have been enhanced with the advent of genomics and modern laboratory technologies. The 2002 unveiling of the *A. gambiae* genome has resulted in the dissection of molecular pathways mediating parasite infection in the mosquito, and in turn the generation of genetically modified, parasite-resistant vectors (Holt et al. 2002; Dong et al. 2011). Furthermore, studies have revealed that mosquitoes harbor a complex internal microbiome, and these microbes play a crucial role in mediating host-pathogen interactions (Dong, Manfredini, and Dimopoulos 2009; Bahia et al. 2014). The combination of these advances in vector biology has resulted in a slew of new vector-based malaria interventions geared towards the goal of global disease elimination.

Figure 1.2: Vector-based Malaria Interventions

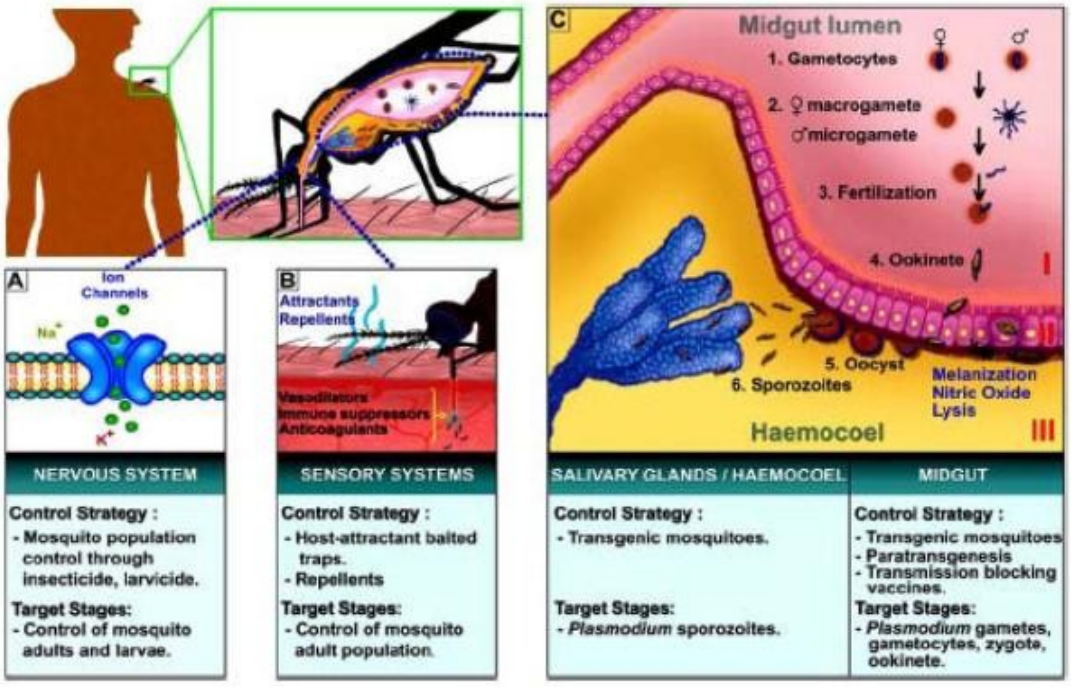

Figure 1.2: Vector-based malaria interventions. From Ramirez *et al*. (2009). *A*. Insecticides used to kill larvae and adults. *B*. Population control by luring and trapping adults. Host-seeking adults are deterred by chemical repellents. *C*. *Plasmodium* parasites targeted for killing in adult females within the midgut or hemolymph (haemocoel). Killing is accomplished by exploiting the mosquito immune system (transgenic mosquitoes) or manipulating mosquito symbionts to produce anti-*Plasmodium* effectors. Transmission blocking vaccines administered to humans block parasite infection in the mosquito.

1.3 Anopheles are world-wide malaria vectors

The *Anopheles* genus of mosquitoes is comprised of nearly 500 species of which only around 8-10% are capable of vectoring malaria parasites

(Collins and Paskewitz 1995). *A. gambiae* is the most well-studied species and primary vector in sub-Saharan Africa. Other species, such as *A. darlingi* and *A. stephensi*, are important vectors in South America and Southeast Asia, respectively (Sinka et al. 2012). For a map of global malaria vectors, see Figure 1.3 (Sinka et al. 2012). There are various *Anopheles* sub-species and reproductively isolated genetic forms that contribute to the complexity of the genus (Lee et al. 2013; Lefèvre et al. 2009). For instance, *A. gambiae* is actually a species complex comprised of many cryptic sub-species that overlap geographically yet remain genetically distinct. It is hypothesized that genetic adaptations to different environments drives *Anopheles* speciation, although other factors undoubtedly contribute to the evolution of the genus (Caputo et al. 2014). In spite of these niche-adaptive differences, *Anopheles* mosquitoes undergo a similar life cycle. Adult females are anautogenous and require a blood meal to produce eggs. Approximately 48-72 hours post-blood meal, gravid females will oviposit approximately 50-200 eggs in a suitable, aqueous environment. *Anopheles* eggs are only partially resistant to desiccation, thus the local environment must remain moist for eggs to be viable. The majority of eggs will eclose within 3 days of oviposition, although temperature variability can contribute to hatch times ranging from 2-30 days or longer. Larvae emerge from the eggs and cycle through 4 developmental stages (L1, L2, L3, L4) that can range from 5-14 days (Prevention 2014b). After the L4

stage, larvae pupate and undergo metamorphosis into adults. For a depiction of the mosquito life cycle, see Figure 1.4 (Charlesworth 2014). Both male and female mosquitoes feed on nectar or other sugars for energy. Adult males typically emerge first and swarm above the larval habitat until females emerge at which point mating commences. Although mosquitoes may survive for up to two weeks in the field, upon mating males typically die off whereas

Figure 1.3: Global Malaria Vectors

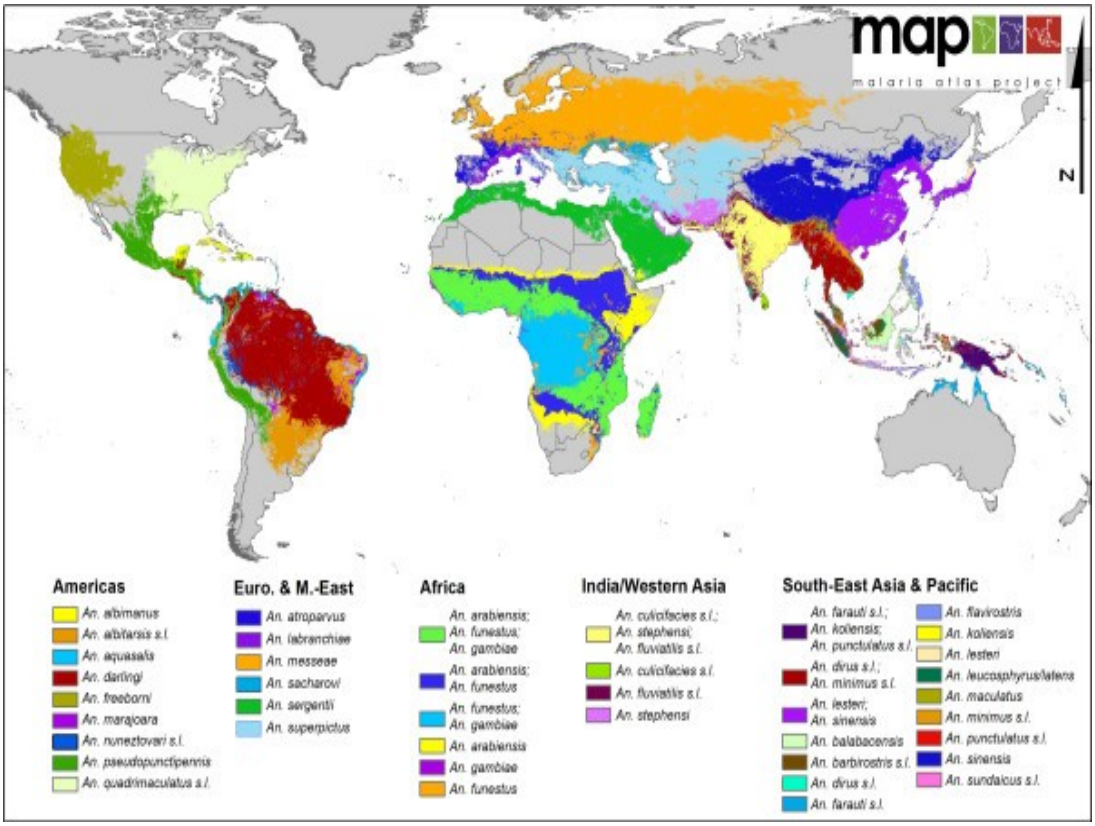

Figure 1.3: *Dominant global malaria vectors*. From Sinka *et al*. (2012). Mosquitoes in the genus *Anopheles* are vectors of human malaria. Colored regions depict distribution of dominant vector species across the world.

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females go in search of blood meal. Females may take more than one blood meal during their life span, and these additional blood meals is when transmission of malaria parasites can occur (Prevention 2014c; Prevention 2014b).

Figure 1.4: **The Mosquito Life Cycle**

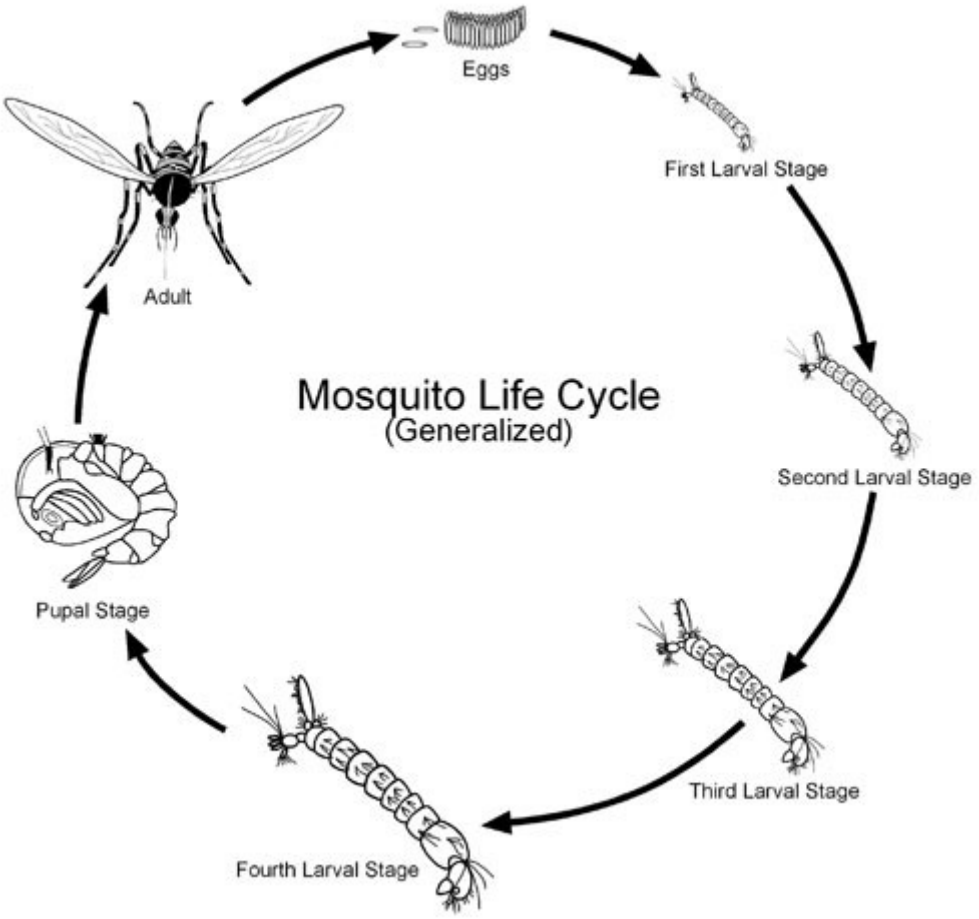

Figure 1.4: *The mosquito life cycle*. Adapted from Scott Charlesworth, Purdue University (2014). Adult females lay eggs in an aqueous environment. After a period of days, larvae eclose from the eggs. Larvae progress through four stages and then pupate. After a few days, adults emerge from the pupae, mate, and then females go in search of a blood meal to acquire nutrients necessary for egg production. Post-blood meal, gravid adult females lay eggs and the cycle begins again. Although the life cycle is generalized, it is applicable to all *Anopheles* mosquitoes. Timing differences between stage transitions are dictated by temperature, nutrient acquisition, and other factors.

1.4 The Plasmodium life cycle

There are five different *Plasmodium* species that infect humans all of which undergo a two host life cycle (CHIN et al. 1965; Ramasamy 2014). *P. falciparum* is undoubtedly responsible for the greatest amount of disease burden and malaria deaths across the world. Falciparum malaria is disproportionately dominant in sub-Saharan Africa in comparison to the rest of the world (Gething et al. 2011). The remaining four species of human parasites, *P. ovale, P. malariae, P. vivax,* and *P. knowlesi* are typically considered less burdensome than *P. falciparum*. However, these four species still constitute an enormous public health problem, and in particular *P. vivax* in South America and Southeast Asia (Gething et al. 2012). Despite differences in developmental time, all *Plasmodium* species have a similar life cycle.

Plasmodium parasites have a complex life cycle comprised of multiple biological forms within the mosquito host, which must successfully cross numerous barriers on their developmental journey through the mosquito host. The life cycle begins when a female mosquito takes an infectious blood meal from a vertebrate host containing the male (microgametocytes) and female (macrogametocytes) sexual forms of the parasite. The change in

temperature from vertebrate to invertebrate hosts triggers microgametocytes to exflagellate and these fuse with macrogametocytes to form a zygote in the midgut of the mosquito. Within 48 hours, the zygote transforms into a motile ookinete that burrows through the midgut epithelium. Upon reaching the basal lamina, the ookinete morphs into an oocyst where asexual reproduction occurs over a period of 7-9 days producing thousands of sporozoites. Approximately 10 or more days post-infectious blood meal, the sporozoites burst out of the oocyst and into the mosquito hemolymph. The hemolymph is an open circulatory system equivalent to mammalian blood, and over a period of days the sporozoites travel to the mosquito salivary gland. Sporozoites enter the salivary glands and are then capable of being transmitted to a susceptible host when the mosquito takes a blood meal (Smith, Vega-Rodríguez, and Jacobs-Lorena 2014; Prevention 2014b). As *Plasmodium* parasites progress through their life cycle, they encounter many challenges inside the mosquito. Parasites must successfully cross physical barriers such as the peritrophic matrix and the midgut epithelium (Cirimotich et al. 2010). The parasite is particularly vulnerable during the transition from ookinete to oocyst, which represents a numerical bottleneck where parasite numbers reach their lowest point in the mosquito (Smith, Vega-Rodríguez, and Jacobs-Lorena 2014). In addition to overcoming physical barriers, parasites must

escape molecular defense mechanisms (Cirimotich et al. 2010). These defenses are coordinated by the insect innate immune system.

1.5 The insect immune system

Over evolutionary time, eukaryotic organisms have faced substantial selective pressure from pathogens. Insects are no exception as they reside in microbe-rich environments throughout their lives. Bacteria, viruses, fungi, parasites, and even physical damage have influenced the evolution of insect immunity (Buchmann 2014). Physical barriers, such as insect cuticle, are the first line of defense (Dubovskiy et al. 2013). When these physical barriers are compromised, pathogens are capable of exploiting the new niches available to them inside the host. In order to combat these invasions, insects have developed defense mechanisms to antagonize pathogens at the molecular level. A common theme of defense mechanisms is the ability to recognize a pathogen, activate appropriate molecular pathways, produce anti-pathogen effector molecules, and to regulate the response (Cirimotich et al. 2010). Defense responses range from highly antagonistic to tolerant depending on the stimuli. These defense mechanisms represent the immune response.

1.5.1 The mosquito innate immune system

Like other insects, mosquitoes possess an innate immune system. The innate immune system is capable of recognizing pathogens and coordinating anti-pathogen defenses (Cirimotich et al. 2010). Although the innate immune system is fully capable of defending against pathogens, it does not form long-lasting protective immunity like the adaptive immune system of vertebrates (Dempsey, Vaidya, and Cheng 2003). Nevertheless, the mosquito immune system is extremely complex and it has the ability to recognize diverse pathogens. The innate immune system has both cellular (hemocytes) and humoral (secreted anti-pathogen effectors) components that dictate the course of an infection. Careful dissection of mosquito immunity has resulted in opportunities to exploit anti-pathogen defenses in order to interrupt *Plasmodium* infection. It is widely accepted that manipulating the mosquito vector to cure or prevent *Plasmodium* infection will effectively break the cycle of transmission to humans.

Our understanding of the mosquito immune system has been guided by seminal research on the *Drosophila* immune system. *Drosophila* has proven an excellent model for the study of genomics due to an array of molecular tools, ease of rearing, and short generation time (Kounatidis and

Ligoxygakis 2012). The publication of the *Drosophila* genome in 2000 has resulted in a meticulous dissection of the fly innate immune system (Adams et al. 2000). Although *Drosophila* are distantly related to mosquitoes, their immune systems are similar enough that direct comparisons can be made between genetic orthologues (Zdobnov et al. 2002). Thus, *Drosophila* has served as a guide for our understanding of the *Anopheles* innate immune system (Adams et al. 2000; Holt et al. 2002; Zdobnov et al. 2002). Comparisons between the immune systems of these two flies have resulted in both synonymy as well as stark differences. Although *Drosophila* is an inherently more tractable model system, *Anopheles* are of public health importance and thus a great body of work has gone into dissecting the mosquito immune system.

The ability to rear both mosquitoes and parasites in the laboratory has provided researchers with the tools to study all aspects of vector biology (Das, Garver, and Dimopoulos 2007). Although vector competence for *Plasmodium* varies between mosquitoes, the narrow host range of the parasite has permitted the use of multiple *Anopheles* species in the laboratory. This has also resulted in distinct laboratory lines of mosquito that display varying susceptibility to parasite infection, providing further tools to dissect the immune response (Stephanie Blandin et al. 2004; Volz et al. 2005). Advanced genetic techniques have allowed the generation of transgenic *Anopheles* for

both basic research and applied purposes (Bernardini et al. 2014; Dong et al. 2011; Pike et al. 2014). The utilization of non-human *Plasmodium* species, such as *P. berghei*, has permitted research to continue in places where access to human parasites is limited (Mendes et al. 2008). Information gleaned from this work has provided new targets and tools for researchers to use in the battle against malaria.

1.5.2 Pathogen associated molecular patterns and pattern recognition

The innate immune system is the key mediator of interactions between mosquitoes and the pathogens that infect them (Clayton, Dong, and Dimopoulos 2014). These interactions are made possible through pattern recognition, the ability to discriminate between different pathogens by recognition of unique microbial ligands (Cirimotich et al. 2010; Clayton, Dong, and Dimopoulos 2014). Pattern recognition receptors (PRRs) recognize microbial pathogen-associated molecular patterns (PAMPs), and these PRRs then activate intracellular immune signaling cascades (Cirimotich et al. 2010). Many different classes of PRRs exist and these are found across the immune pathways (Volz et al. 2005; Dong and Dimopoulos 2009; Meister et al. 2009; Kurata 2014; Christophides et al. 2002). The first step of regulation

involves the identification of pathogens as non-self. Pathogens often share moieties with subtle structural differences that are detected by various classes of PRRs (Cirimotich et al. 2010; Clayton, Dong, and Dimopoulos 2014; Dong and Dimopoulos 2009; Dong, Taylor, and Dimopoulos 2006; Dong, Cirimotich, et al. 2012). PAMPs are detected by cell-bound, soluble, or intracellular PRRs (Cirimotich et al. 2010; Bosco-Drayon et al. 2012). Upon recognition of the target ligand, some PRRs activate immune signaling pathways whereas others inhibit the immune response (Meister et al. 2009; Zaidman-Rémy et al. 2006; Bosco-Drayon et al. 2012). These competing signals are important as they ensure that the immune system will neither become overactive nor indiscriminately antagonize microbes. Many commensal microbes inhabit the mosquito midgut and other organs, and an overactive immune response can cause harm to the mosquito (Minard, Mavingui, and Moro 2013). In the case of bacteria, it remains unclear if the mosquito immune system is capable of distinguishing between individual species of bacteria (Stathopoulos et al. 2014). However, the immune system is capable of differentiating between gram positive and negative bacteria which demonstrates a high degree of specificity (De Gregorio et al. 2002; Meister et al. 2005; Meister et al. 2009). Furthermore, the immune response to different *Plasmodium* parasites is markedly different (Dong et al. 2006). The ability to identify invading pathogens is merely the first step in the immune process.

1.5.3 Immune signaling pathways

Upon the recognition of PAMPs, PRRs ultimately activate intracellular signaling cascades of the innate immune system. The translocation of transcription factors to the nucleus regulates the production of immune gene mRNAs which code for PRRs, components of anti-pathogen effector mechanisms and immune activity regulators (Cirimotich et al. 2010). Although seemingly simple, the actual mosquito immune response to a pathogen is quite complex. Despite this complexity, molecular approaches have enabled the dissection of the pathways involved in controlling mosquito innate immunity. Studies on *Drosophila* immune pathways have guided the dissection of their orthologues in the mosquito immune system (Zdobnov et al. 2002). Researchers have used a combination of functional genomics, transcriptomics, and proteomics to characterize the major immune signaling pathways in the mosquito. These studies have resulted in an understanding of the major immune pathways implicated in anti-pathogen defense. For a graphical depiction of the major immune pathways, see Figure 1.5 (Jose Luis Ramirez et al. 2012).

1.5.3.1 The Toll pathway

Upon activation of PRRs that can bind to microbial PAMPs, two main signaling pathways launch effective anti-pathogen immune responses (Cirimotich et al. 2010). The first pathway is known as the TOLL pathway and is an intracellular signaling pathway downstream of the membrane bound TOLL receptor (Lemaitre et al. 1996). Originally described in *Drosophila*, the TOLL pathway is involved in guiding dorsoventral patterning during fly development (Lemaitre et al. 1996). However, the TOLL pathway also functions as an immune pathway responsive to gram-positive bacteria, viruses, *Plasmodium berghei,* and fungi (Cirimotich et al. 2010). PRRs recognize gram-positive bacteria or fungi and initiate extracellular serine protease signaling cascades that culminate in the binding of the ligand Spätzle to the TOLL receptor (Lemaitre et al. 1996; Valanne, Wang, and Rämet 2011; Ming et al. 2014). Serine protease cascades also play other roles in immune responses (see Section 1.5.4), providing signal amplification that leads to the activation of anti-pathogen effector mechanisms such as melanotic encapsulation (Law et al. 2006; Suwanchaichinda and Kanost 2009; Zou et al. 2010). The intracellular TOLL pathway is activated by the activation and binding of Spätzle, and this leads to the activation of an intracellular signaling cascade which in turn results in the degradation of the negative regulator of the pathway known as Cactus (Cirimotich et al. 2010).

Cactus sequesters the NF-kappaB like transcription factor Rel1, and proteosomal degradation of Cactus allows Rel1 to translocate to the nucleus and initiate anti-pathogen defense responses (Cirimotich et al. 2010). Rel1 mediates the production of many anti-microbial effectors, but only a modest number have been described in great detail (Dong et al. 2006; Meister et al. 2005; Shin, Zou, and Raikhel 2011). Global analysis of the mosquito transcriptome has revealed that Rel1 regulates transcript abundance of non-immune genes in multiple functional groups (Dong et al. 2006; Garver, Dong, and Dimopoulos 2009). This indicates that the Rel1 transcriptome is complex and involved in many non-immune cellular processes. There are actually a total of 11 TOLL family genes in *Anopheles*, but aside from the seminal TOLL pathway the role of these related receptors in anti-pathogen defense responses is largely unexplored (Imler and Zheng 2004).

1.5.3.2 The IMD pathway

The second main signaling pathway that launches anti-pathogen immune responses is the immunodeficiency (IMD) pathway (Myllymäki, Valanne, and Rämet 2014; Garver et al. 2012; Garver, Dong, and Dimopoulos 2009). This pathway was also initially described in *Drosophila* and is equated to the tumor necrosis factor (TNF) signaling pathway in humans (Kaneko

and Silverman 2005; Cirimotich et al. 2010). The IMD pathway is involved in defense against gram-negative bacteria and *Plasmodium falciparum* (Cirimotich et al. 2010). Unlike the TOLL pathway, the link between pattern recognition receptor and pathway activation is direct as cell surface bound peptidoglycan recognition proteins (PGRPs) mediate activation of the IMD pathway (Kurata 2014; Meister et al. 2009). PGRP binding of bacterial peptidoglycan activates an intracellular signaling cascade that branches into two distinct directions (Cirimotich et al. 2010). Like the TOLL pathway, the main branch of the IMD pathway also exerts its anti-pathogen defenses through the translocation of an NF-kappaB like transcription factor, REL2, to the nucleus (Cirimotich et al. 2010). Rel2 exists in two states, a long form (REL2-f) and short form (REL2-s) (Meister et al. 2005). Upon activation of the IMD pathway, Caspar is degraded which frees REL2-f and its translocation to the nucleus results in the transcription of anti-pathogen effector genes (Cirimotich et al. 2010). Unlike REL2-f, REL2-s expression is constitutive and it continuously translocates to the nucleus where it mediates basal levels of immune stimulation (Meister 2006; Cirimotich et al. 2010). The two species of REL2 seem to regulate the production of different effector molecules, although it remains unclear how much cross regulation occurs (Meister 2006). Studies have shown that REL2 controls the mosquito midgut microbiota, whereas the Toll pathway and its transcription factor Rel1 does

not (Jose L Ramirez and Dimopoulos 2010; Dong, Manfredini, and Dimopoulos 2009). The midgut microbiota are predominantly composed of gram-negative bacteria, and it has been hypothesized that the IMD pathway controls bacterial proliferation stimulated by an influx in nutrients post blood meal (Dong, Manfredini, and Dimopoulos 2009). The implication of the midgut microbiota in mediating IMD pathway activation and anti-pathogen defense is discussed in Section 1.8.

1.5.3.3 The JNK pathway

The other branch of the IMD pathway bares similarities to the mammalian c-jun N-terminal kinase (JNK) pathway. JNK is a member of the mitogen-activated protein kinase (MAPK) family that is highly conserved between mammals and insects (Horton et al. 2011). JNK exerts its effects by activating the transcription factor AP-1, and is involved in multiple physiological processes including apoptosis, cell differentiation, stress response, and immunity (Cirimotich et al. 2010). While it remains unclear if this branch of the IMD pathway is activated independently of the Rel2 branch, studies in *Drosophila* suggest that JNK signaling is transient and negatively regulated through activation of the NF-kappaB-like branch (Park et al. 2004). Despite this finding, studies in the mosquito have shown that the

JNK pathway plays an essential role in regulating oxidative stress, hemocyte immune cell differentiation, and the production of anti-microbial effectors (Garver, de Almeida Oliveira, and Barillas-Mury 2013; Cirimotich et al. 2010; Jaramillo-Gutierrez et al. 2010; Jose Luis Ramirez, Garver, et al. 2014). In the mosquito midgut, activation of JNK promotes epithelial cell nitration in response to damage of the midgut epithelium (G. de A. Oliveira, Lieberman, and Barillas-Mury 2012). Nitration is an important process that tags damaged cells as well as pathogens in order to make them "visible" to the mosquito immune system. JNK activation also results in the production of cytokines that in turn are capable of activating the JAK-STAT pathway (Amoyel and Bach 2012). The JNK pathway is complex and further studies are needed to identify molecular determinants responsible for activation of this branch downstream of IMD.

1.5.3.4 The JAK-STAT pathway

Another immune pathway in the mosquito is the Janus-kinase/signal transducers and activation of transcription (JAK-STAT) pathway. The JAK-STAT pathway is conserved between mammals and insects, and has roles in development, stem-cell maintenance, cellular proliferation, hemocyte differentiation, and immunity (Souza-Neto, Sim, and Dimopoulos 2009;

Agaisse and Perrimon 2004; Amoyel and Bach 2012; Cirimotich et al. 2010). In flies, the JAK-STAT pathway is activated by the binding of the protein unpaired (Upd) to the extracellular region of a transmembrane receptor called Dome. This process ultimately results in STAT translocating to the nucleus where it induces the expression of many genes including those involved in anti-pathogen defense (Hombría and Brown 2002; Arbouzova and Zeidler 2006). The negative regulator PIAS controls pathway activation by blocking STAT transcription. The pathway is probably activated by PAMPs in the midgut, but JAK-STAT activation in the fat body may be due to a systemic signal amplification (Pastor-Pareja, Wu, and Xu 2014). Recently, a secreted factor called Vago has been shown to activate the JAK-STAT pathway in response to infection (Paradkar et al. 2012). In mosquitoes, the pathway is implicated in anti-viral, anti-fungal, and anti-*Plasmodium* defense (Cirimotich et al. 2010). JAK-STAT regulates the production of nitric oxide synthase (NOS) that has diverse functions in anti-pathogen defense (Hillyer and Estévez-Lao 2010; Luckhart et al. 1998; Ramos-Castañeda et al. 2008). Nevertheless, the mechanisms underlying JAK-STAT-mediated anti-pathogen defenses in the mosquito are largely unexplored.

1.5.4 Serine proteases in immunity

The mosquito genome encodes dozens of SPs and serine protease homologues (SPHs) that regulate extracellular signaling and immune defenses (Gorman and Paskewitz 2001; Suwanchaichinda and Kanost 2009; Gulley, Zhang, and Michel 2013). SPs participate in a variety of physiological process including blood digestion, immunity, and wound healing (Gubb et al. 2010). Perhaps their most important ability is the amplification of signals, typically in highly regulated SP cascades. Signal amplification allows the mosquito to turn local immune signaling into a systemic response in preparation for invading pathogens. This ability offers the host an advantage of a rapid immune response, however, over activation of a SP cascade can be highly detrimental to the host's survival (Michel et al. 2005). Serine protease

Figure 1.5: The *Anopheles* Innate Immune Pathways

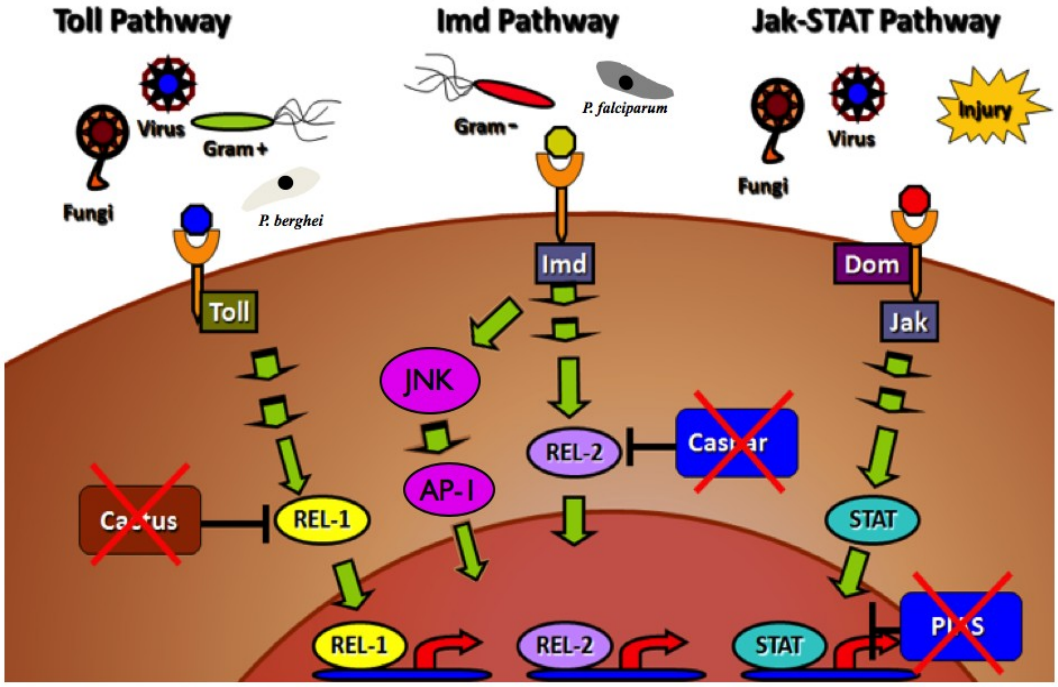

Figure 1.5: *The major Anopheles immune pathways*. Adapted from Ramirez *et al*. (2012). *Left*, the Toll pathway controls fungi, viruses, gram positive bacteria, and *P. berghei*. The transcription factor Rel-1 mediates the production of Toll pathway anti-pathogen effector molecules and Cactus negatively regulates Rel-1. *Center*, the IMD pathway controls gram negative bacteria and *P. falciparum*. The transcription factor Rel-2 mediates the production of IMD pathway anti-pathogen effector molecules and Caspar negatively regulates Rel-2. The IMD pathway can also branch into the JNK pathway, which mediates anti-pathogen defense and other physiological processes through the transcription factor AP-1. *Right*, the JAK-STAT pathway controls fungi, viruses, and injuries. The STAT transcription factor mediates production of JAK-STAT pathway effector molecules, and PIAS negatively regulates STAT.

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inhibitors (SRPNs) predominantly function as negative regulators of SP cascades (Law et al. 2006). The advantage of a protease cascade is that it creates multiple checkpoints to regulate the immune response to avoid over

activation. The complexity of SP cascades and SRPNs makes them difficult to study, but despite this researchers have been able to characterize some SPs and SRPNs involved in mosquito immunity.

One of the larger families of serine proteases are clip-domain serine proteases (CLIPs), which are named after their structural resemblance to a paper clip (Jang, Nam, and Lee 2008). This family was originally described in blood clotting of the horseshoe crab (Muta, Oda, and Iwanaga 1993). In the mosquito, five families (CLIPs A-E) exist (Gorman and Paskewitz 2001). CLIPs are regulators of immune signaling cascades, although some may act as PRRs of danger signals (Ming et al. 2014). Most of the research has focused on CLIPA and CLIPB family members. For instance, CLIPA2 has been discovered to bind to microbial surfaces and act as a negative regulator of the complement-like response in mosquitoes (Yassine et al. 2014). Alternatively, SPCLIP1 is a non-catalytic positive regulator of complement-like factor accumulation on microbial surfaces (Povelones et al. 2013). Other CLIPs are involved in regulating the prophenyloxidase (PPO) system or melanization, an important immune response involved in wound healing and anti-pathogen defense (Cerenius, Lee, and Söderhäll 2008). CLIPB9 is a positive regulator of melanization in the mosquito (An et al. 2011). CLIPB14 and CLIPB15 have structural similarity to PPO enzymes in other organisms, and both are secreted by hemocytes in response to pathogen challenge (Volz

et al. 2005). However, neither of these SPs are essential for melanization in certain lab strains of mosquitoes, suggesting that CLIP immune functions can vary between mosquitoes (Volz et al. 2005). Even less is known about the CLIPC, D, and E families. CLIPs are an integral part of mosquito immune processes, and ongoing research of this SP family will aid in the dissection of immune defense mechanisms.

Serine protease inhibitors (SRPNs) participate in a wide range of processes in the mosquito. Structural modeling indicates that most SRPNs are inhibitory (Suwanchaichinda and Kanost 2009). The active site of a SRPN acts as a "bait" for its target SP. Upon SP cleavage of the bait, the SRPN binds the SP irreversibly inactivating it (Suwanchaichinda and Kanost 2009; Law et al. 2006). Only a few mosquito SRPNs have been characterized, and our understanding of their functions is limited. The many processes regulated by SRPNs contributes to their ability to function as pathogen host factors or restriction factors. Some SRPNs are involved in regulating the mosquito melanization response, such as SRPN2. SRPN2 is known to inhibit the SP CLIPB9, which itself is a positive regulator of melanization (Michel et al. 2005; An et al. 2011). SRPN6 is involved in pathogen killing in *A. stephensi*, whereas in *A. gambiae* it may participate in pathogen clearance (Abraham et al. 2005). This suggests SRPN function may be dependent on the genetic composition of the mosquito species. SRPN6 is also activated by gram

negative bacteria, highlighting the role of SRPNs across multiple immune pathways (Eappen, Smith, and Jacobs-Lorena 2013). Uncharacterized mosquito SRPNs may play important roles in an immunity and this warrants further investigation in the responses they govern.

1.6 The complexity of the immune response

Studies of the mosquito innate immune system have progressed steadily since the 2002 publication of the *Anopheles* genome. Researchers have been able to describe the major immune pathways and their roles in regulating defense against pathogens. These studies have resulted in major advances such as genetically modified *Anopheles* that are resistant to *Plasmodium* infection (Dong et al. 2011). Despite advances, the field of vector biology is still unraveling the complexity of the mosquito immune system. The *Anopheles* genome contains many uncharacterized immune genes, and some are regulated upon *Plasmodium* infection (Dong et al. 2006). These could become new targets in strategies exploiting the mosquito immune system to interrupt parasite transmission.

Drosophila research has provided much needed guidance to researchers dissecting the mosquito immune system. Unlike mosquitoes, *Drosophila* is a highly tractable genetic model. The use of the GAL4/UAS

system to study gene expression as well as the ability to efficiently knock out genes makes it a superior model to *Anopheles* in this regard (McGuire, Roman, and Davis 2004). *Anopheles* gene functions have primarily been characterized through use of RNA interference (RNAi), a process used to deplete transcript abundance in order to study gene function (Shin, Kokoza, and Raikhel 2003). A drawback of using RNAi is that it makes studying complex pathways challenging as the amount of work required to knock down multiple steps in a given pathway becomes unreasonable. Nevertheless, RNAi has been critical in the identification of genes involved in anti-*Plasmodium* defense.

Our understanding of the *Anopheles* immune pathways is likely oversimplified. At first glance the immune pathways appear to be streamlined and responsive to discrete stimuli. However, transcriptomics has revealed that the transcription of many genes is regulated by more than one immune pathway, and this is further complicated by genetic differences between laboratory strains of mosquito (Garver, Dong, and Dimopoulos 2009). Aside from regulating the production of anti-pathogen effector molecules, the immune pathways regulate the transcript production of other genes belonging to diverse functional groups, some of which have anti-pathogen activity (Pike et al. 2014). Further studies are needed to unravel

the complexity underlying mosquito innate immunity and to explore the function of uncharacterized genes.

1.7 The immune response to Plasmodium

Plasmodium parasites are antagonized by the mosquito immune system from their ingestion in a blood meal through their invasion of the mosquito salivary glands. When a female mosquito takes an infectious blood meal from a vertebrate host, the microgametocytes and macrogametocytes fuse to form a motile ookinete in the midgut. Prior to invading the midgut epithelium, the ookinete must escape from the peritrophic matrix (PM). The PM is composed of chitin and glycoproteins that separate the blood meal from the midgut epithelium, providing a semi-permeable barrier for a regulated exchange of molecules (Dinglasan et al. 2009). The components of the PM are secreted following a blood meal, and the PM is fully matured around 16-24 hours post blood meal (Dinglasan et al. 2009). Motile ookinetes escape the PM by utilizing a chitinase to break down the barrier and those parasites that are incapable of escaping this first line of defense perish (Langer and Vinetz 2001). By about 48 hours, the ookinetes that have escaped the PM have invaded the midgut epithelium. This invasion induces a broad response in the mosquito including the production of anti-microbial effectors,

cytoskeletal rearragenments, melanization, and other metabolically intense processes (Vinetz 2005). This response is presumably to antagonize the parasite, repair damage to the epithelium, and to mitigate the overall harm caused by event. Upon reaching the basal lamina, *Plasmodium* reaches a point in its life cycle that is a numerical bottleneck with few parasites advancing from ookinetes to viable oocysts (Smith, Vega-Rodríguez, and Jacobs-Lorena 2014). The decrease in the total parasite load up to this point is due to multiple factors, but none more important than the mosquito immune response. Natural *P. falciparum* infections in field caught mosquitoes are frequently found to have fewer than five oocysts (Sinden, Alavi, and Raine 2004; Pringle 1966). Therefore, targeting the parasite in the mosquito at this stage of its life cycle is an attractive option to exploit the vulnerability of a low parasite load. For a depiction of the bottleneck in parasite numbers, see Figure 1.6 (Kafatos Lab 2014).

The surviving oocysts enter multiple rounds of asexual reproduction in a relatively protected environment. In some strains of mosquitoes, defense against oocysts involves melanization or the encapsulation of the oocyst along with reactive oxygen species production. At approximately 10 or more days post-infectious blood meal, asexual reproduction ceases and sporozoites burst out of the oocyst into the mosquito hemolymph. The hemolymph is an open circulatory system equivalent to mammalian blood that contains many

immune effectors. Co-circulating in the hemolymph are hemocytes, which are types of cells that participate in the immune response (Jose Luis Ramirez, Garver, et al. 2014). The fat body of the mosquito, equivalent to the mammalian liver, is also in direct contact with the hemolymph and serves as the source of many anti-microbial effectors (Manfruelli et al. 1999). Although the mosquito does mount an immune response to sporozoites, thousands end up reaching and invading the salivary glands (Pinto, Kafatos, and Michel 2008). Finally, the parasite is ready to be transmitted to a susceptible host when the mosquito takes its next blood meal.

Plasmodium parasites take a perilous journey through the mosquito that lasts many days. The bottleneck in parasite numbers is in large part

Figure 1.6: Bottleneck of *Plasmodium* in the Mosquito Midgut

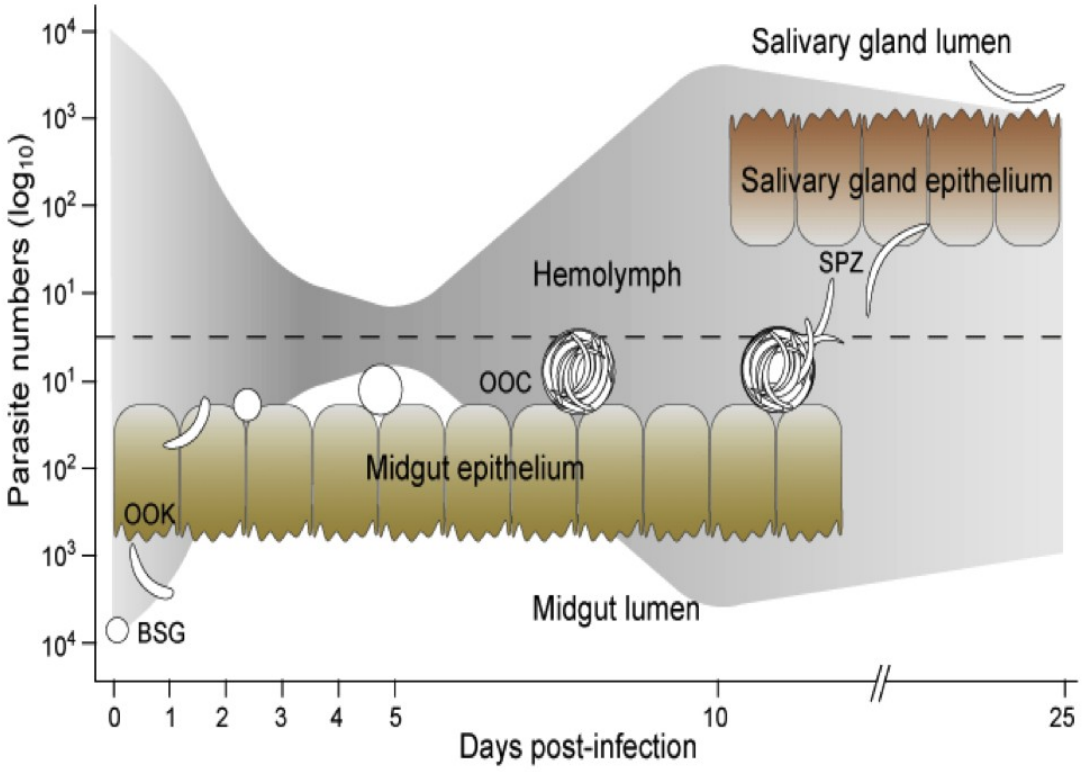

Figure 1.6: The bottleneck of Plasmodium parasite numbers in the midgut. From the Kafatos Lab (Imperial College, London). Website: http://kafatos.openwetware.org. (2014). Total parasite load in *Anopheles* undergoes a drastic reduction upon ookinete traversal of the midgut epithelium and the formation of oocysts. The decrease in parasite numbers is primarily attributable to *Anopheles* anti-*Plasmodium* defenses.

attributable to the mosquito immune response. Therefore, it is not surprising that a great deal of research has gone into dissecting the mosquito immune system from the standpoint of developing tools to combat the parasite in the mosquito. Researchers have used genomics, global transcriptomics,

proteomics, and other strategies to identify the key immune pathways and effector molecules that comprise anti-*Plasmodium* defenses.

1.7.1 Anti-Plasmodium effector molecules and mechanisms

Studies have probed the transcriptional profile of mosquitoes at different stages of the *Plasmodium* life cycle, especially in the midgut during ookinete invasion. Effectors have been classified mainly through RNAi-based transcript depletion that results in increased levels of *Plasmodium* infection. Further characterization has associated some of these effector molecules with specific immune pathways and processes. Although there are a number of anti-*Plasmodium* effector molecules, only the most pertinent ones are covered in this section. For an overview of anti-*Plasmodium* defense in the midgut, see Figure 1.7 (Cirimotich et al. 2010).

Thioester-containing protein 1 (Tep1) one of the most well studied anti-*Plasmodium* effector molecules. Tep1 participates in spectrum of immune responses including phagocytosis, parasite lysis, and melanization (Stephanie Blandin et al. 2004; Garver et al. 2012; Yassine, Kamareddine, and Osta 2012). Infection studies have shown that it controls both *P. berghei* and *P. falicparum* in the mosquito midgut (Garver, de Almeida Oliveira, and Barillas-Mury 2013; Garver, Dong, and Dimopoulos 2009). Tep1 was

identified as the mosquito orthologue of C3 in the human complement system (Stephanie Blandin et al. 2004). Recent work has established the role of Tep1 in a highly regulated complement-like process in the mosquito, in which Tep1 is deposited on the surface of pathogens (Yassine et al. 2014). Tep1 expression is strongly regulated by the IMD pathway (Garver, Dong, and Dimopoulos 2009). Although there are other Teps in the mosquito, their role in anti-*Plasmodium* defense is not known.

Another important anti-*Plasmodium* effector molecule is leucine-rich domain containing protein 7 (LRRD7). Leucine-rich domains are often associated with pattern recognition. The expression of LRRD7 is under the control of the IMD pathway and it is effective in defense against *P. falciparum* (Garver, Dong, and Dimopoulos 2009; Garver et al. 2012). A related leucine-rich immune protein (LRIM1) has been shown to control *P. berghei* infection, suggesting differences in pattern recognition between proteins containing leucine-rich domains (Meister et al. 2005). Research has shown that Tep1 forms complexes with leucine-rich proteins on the surface of parasites, indicating these molecules may be involved in the complement-like process (Baxter et al. 2010). However, the underlying mechanism behind this finding is not well understood and there are possibly many more interacting partners of leucine-rich proteins.

Recent work has demonstrated that fibrinogen-related proteins (FBNs) participate in anti-*Plasmodium* defense. There are a total of 59 FBNs in the mosquito genome, and these are presumably pattern recognition proteins capable of mediating anti-pathogen defense (Dong and Dimopoulos 2009). In other model systems, FBNs participate in phagocytosis and complement activation (Dong and Dimopoulos 2009). In the mosquito, a number of FBNs are induced by either bacterial or *Plasmodium* challenge (Garver, Dong, and Dimopoulos 2009; Dong and Dimopoulos 2009; Cirimotich et al. 2010). FBN9 is regulated by the IMD pathway, and it is a potent anti-*P. falciparum* effector molecule associated with ookinetes in the midgut (Garver, Dong, and Dimopoulos 2009; Garver et al. 2012). The mechanism by which FBN9 antagonizes *P. falciparum* is unknown.

Figure 1.7: *Anopheles* Anti-*Plasmodium* Defenses in the Midgut

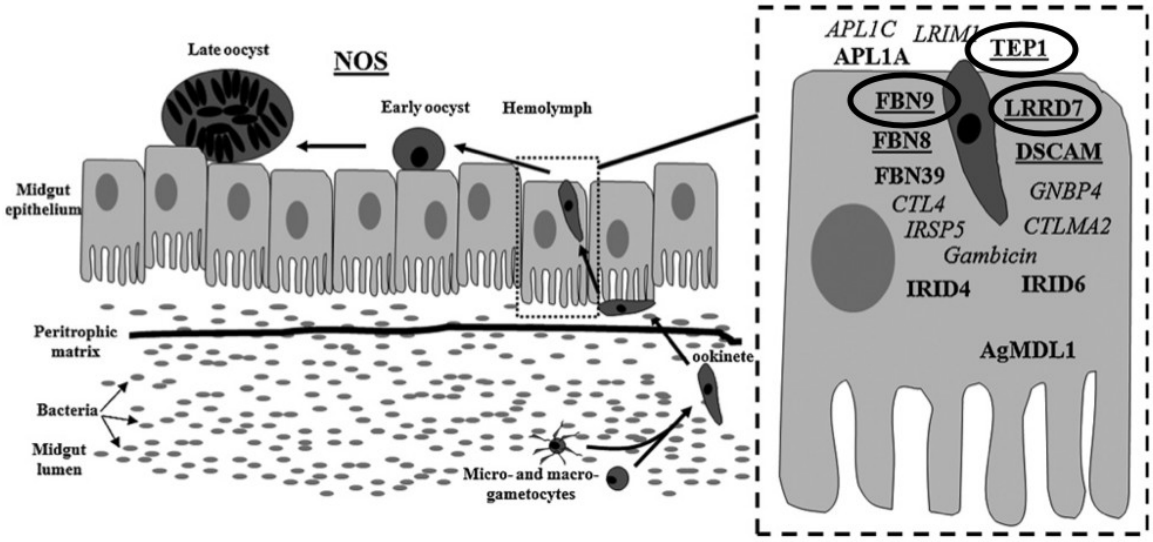

Figure 1.7: *Anopheles anti-Plasmodium defenses in the midgut*. Adapted from Cirimotich *et al*. (2010). *Left*, *Plasmodium* ookinetes escape through the peritrophic matrix, invade the luminal side of the midgut epithelium, traverse basolaterally, and form oocysts upon reaching the basement membrane. *Right*, anti-*Plasmodium* effector molecules that antagonize parasites. The black circles indicate the most potent anti-*P. falciparum* effectors known, and their production is regulated by the IMD pathway.

1.7.2 Anti-Plasmodium immune pathways

Laboratory infection models have established the important immune pathways mediating anti-*Plasmodium* defenses. While the TOLL pathway has been shown to suppress infection with the rodent malaria parasite *P. berghei*, the IMD pathway is associated with anti-*P. falciparum* defense (Dong et al. 2006; Garver, Dong, and Dimopoulos 2009; Cirimotich et al. 2010). This important finding further highlights stark differences in studies

using *P. berghei* as a model of *P. falciparum* infection. The JNK pathway controls nitration that occurs during the invasion of the midgut epithelium by *P. berghei*, although JNK-mediated epithelial cell nitration has not been studies in the context of *P. falciparum* infection (G. de A. Oliveira, Lieberman, and Barillas-Mury 2012; Garver, de Almeida Oliveira, and Barillas-Mury 2013). The JAK-STAT pathway also mediates defense against both *P. berghei* and *P. falciparum* at a later stage of infection, and it has been shown to control *P. vivax* infections in *A. aquasalis* (Cirimotich et al. 2010; Bahia et al. 2011). However, the mechanisms underlying JAK-STAT-mediated defense against *Plasmodium* are largely unexplored.

The IMD pathway may be the most important immune pathway mediating anti-*P. falciparum* defenses. Silencing of the negative regulator Caspar activates the Rel2 branch of the IMD pathway, and controls *P. falciparum* infection (Garver, Dong, and Dimopoulos 2009). IMD-mediated defenses against *P. falciparum* have been observed in *A. gambiae*, *A. stephensi*, and *A. albimanus* indicating that *P. falciparum* defense may be independent of mosquito species (Garver, Dong, and Dimopoulos 2009). Unlike the Toll pathway, the IMD pathway is not particularly effective at controlling *P. berghei* demonstrating the *P. falciparum*-specificity of the IMD pathway (Garver, Dong, and Dimopoulos 2009). Much of the IMD anti-*Plasmodium* response occurs through Rel2-mediated anti-*Plasmodium*

effectors FBN9, LRRD7, TEP1, and other defense proteins (Cirimotich, Ramirez, and Dimopoulos 2011; Jose Luis Ramirez et al. 2012; Meister et al. 2009; Dong, Manfredini, and Dimopoulos 2009; Cirimotich et al. 2010; Cirimotich et al. 2011). The importance of Rel2-mediated defense is supported by the generation of transgenic *A. stephensi* that are resistant to *P. falciparum* infection (Dong et al. 2011). These mosquitoes harbor a transgene containing Rel2 under the control of a blood meal inducible promoter. When a transgenic mosquito takes a blood meal, a transient yet dramatic increase in Rel2 expression mediates strong defenses against *P. falciparum* infection (Dong et al. 2011).

Although Rel2-mediated defenses are effective, there are other defenses against *P. falciparum* that have yet to be explored in depth. The use of different laboratory mosquito strains has complicated our understanding of anti-*Plasmodium* defenses. For instance, resistant strains of *A. gambiae* have a strong melanization response to *P. falciparum,* but susceptible strains may be reliant on the IMD-mediated defenses. In some laboratories, it is not possible to work with *P. falciparum* so *P. berghei* has been used its place. *P. berghei* infection models have proven an essential tool in dissecting mosquito immunity, but further studies are required to determine the relevance to *P. falciparum* infection. Nevertheless, our understanding of anti-*Plasmodium*

defenses in *Anopheles* has advanced significantly, and offers targets that can be exploited to interrupt parasite transmission in the field.

1.8 The mosquito microbiome

Host-pathogen interactions are shaped by their environment. In the case of *Plasmodium* infection in *Anopheles* mosquitoes, parasites interact with the mosquito's internal environment. Parasites encounter a rich array of microbes predominantly in the mosquito midgut. The bacteria and fungi residing in the mosquito midgut are referred to as the microbiome. In recent years, there has been a surge of research addressing the role of the microbiome in the mosquito. Recent advances in our understanding of the mosquito microbiome have demonstrated that microbes play a major role in determining the outcome of pathogen infection in the mosquito. Bacteria residing in the mosquito midgut are particularly important for the priming of immune defenses, and some species are determinants of *Plasmodium* infection in the mosquito. Less is known about the role of fungi in immunity and pathogen susceptibility. Nevertheless, characterization of mosquito symbionts has elucidated molecular mechanisms responsible for the inhibition of pathogen infection, and has provided natural microbial candidates for the development of strategies to interrupt disease

transmission. Henceforth, host-pathogen interactions are viewed as tripartite interactions between the mosquito, *Plasmodium* parasites, and the mosquito microbiome.

1.8.1 The mosquito bacteriome

The bacterial microbiome plays an important role in modulating physiological processes in the mosquito including the outcome of pathogen infection. We will refer to all the bacteria that share or occupy space within or on the body of the mosquito as the microbiota. Much like humans, bacteria reside in the digestive organs of mosquitoes as well as other tissues(Chao J, Wistreich GA 1963). The microbes found in the mosquito gut are of particular interest because they share a physical space with invading human pathogens. They therefore have the potential to affect infection success either through direct interactions with pathogenic organisms or by eliciting mosquito immune system signaling.

While our understanding of the factors determining mosquito microbiota composition remains incomplete, important insights are gradually emerging. A meta-analysis of multiple bacterial sampling studies showed that the mosquito microbiota differs to some degree between mosquito species(Minard, Mavingui, and Moro 2013). However, it is also highly

variable between individuals of the same species or population, suggesting that environmental or physiological factors are likely to be important in shaping the bacterial gut microbiota(Osei-Poku et al. 2012; Zouache et al. 2011; Boissière et al. 2012). Mosquito larvae encounter microorganisms in their aqueous environment and these microbes are inevitably introduced into the mosquito gut when larvae consume microbes for food(Minard, Mavingui, and Moro 2013). Similarly, adult mosquitoes can ingest microbes from larval water as they eclose from the pupal casing or as adults during nectar feeding, as flower nectar contains many bacteria commonly found in adult mosquitoes(J M Lindh, Borg-Karlson, and Faye 2008; Shi, Lou, and Li 2010; Alvarez-Pérez, Herrera, and de Vega 2012; Yamada and Yukphan 2008). Differences in the larval or adult environment could therefore influence which microbes are introduced into the mosquito gut. For example, *Anophelines* prefer clear water whereas mosquitoes from a different genus, *Aedes*, utilize a wide variety of water sources for larval development whereas, which may cause differences in the gut microbiomes between species(AN. 1999; Minard, Mavingui, and Moro 2013). Collection location has been reported to be a major determinant of gut microbe composition within species, and it has been suggested that this may largely be due to differences in vegetation type or vertebrate host availability between populations(Boissière et al. 2012; Zouache et al. 2011).

Physiological processes, such as molting, sugar and/or blood feeding, and reproduction also have the potential to exert influence on which microbes are introduced and can persist within the mosquito. As larvae develop, bacteria can persist between larval stages and from larvae to pupae, though it is not clear in all cases whether this is due to transstadial transmission or to re-ingestion of microbes from shared water sources(J M Lindh, Borg-Karlson, and Faye 2008; Favia et al. 2007). During metamorphosis from pupae to adult, the number of bacteria in the gut is markedly reduced, and the composition is also dramatically altered(Moll et al. 2001; Moncayo et al. 2005; Wang et al. 2011; Chavshin et al. 2012; Rani et al. 2009; Dinparast Djadid et al. 2011). Multiple reports indicate shared bacterial species between pupae and adults suggesting that some bacteria may persist during this developmental transition(Chavshin et al. 2012; Rani et al. 2009; Wang et al. 2011). However, it is unclear whether bacteria that persist into adulthood are transstadially transmitted or simply re-acquired after eclosion through adult ingestion of breeding water(J M Lindh, Borg-Karlson, and Faye 2008). As adults, feeding behavior can also dramatically affect the gut microbiota. Sugar feeding has been shown to alter the microbial composition of the gut; in one study a single sterile sugar meal resulted in a slight reduction in bacterial diversity, with a corresponding increase in bacteria from Flavobacteriaceae and a decrease in bacteria from Enterobacteriaceae(Wang

et al. 2011). Blood feeding results in dramatic increases in bacterial number in the female gut, though it also causes a corresponding decrease in bacterial diversity(Pumpuni et al. 1996; Terenius et al. 2012; J. H. M. Oliveira et al. 2011; Wang et al. 2011). Reproduction can also act as an avenue for acquisition of gut bacteria. For example, *Asaia* bacteria can be sexually transmitted from males to females and after sexual transmission are found in the female midgut(Damiani et al. 2008; Favia et al. 2007). There is also evidence that bacteria can be passed vertically from mother to offspring, either through egg-smearing, transovarial transmission or maternal inoculation of larval water(Favia et al. 2007; Mitraka et al. 2013; I. G. Akhouayri, Habtewold, and Christophides 2013). Efforts to catalog the bacteria present in the digestive tracts of field and laboratory mosquitoes from diverse species, populations and collection locales have provided an important initial characterization of the mosquito gut microbiota.

1.8.2 The mosquito's bacteriome modulates Plasmodium infection

The mosquito microbiota has been shown in many studies to be an important determinant of pathogen infection outcome. For example, reduction of the bacteria in the gut through antibiotic treatment results in increased susceptibility to *P. falciparum* infection, and ingestion of certain

bacterial species can result in dramatically reduced infection levels (Dong, Manfredini, and Dimopoulos 2009; Xi, Ramirez, and Dimopoulos 2008; Cirimotich et al. 2011; Bahia et al. 2014; Jose Luis Ramirez et al. 2012; Eappen, Smith, and Jacobs-Lorena 2013; Tchioffo et al. 2013). Interestingly, in some studies, the presence of certain bacterial species in the gut correlated with increased susceptibility to *P. falciparum* infection, suggesting that the effect of gut bacteria on pathogen infection is complex and may depend on the specific composition of the gut microbiota (Boissière et al. 2012; Apte-Deshpande et al. 2012). In order to cause reduced infection levels, bacteria may interact directly and/or indirectly with invading pathogens. One way gut microbes indirectly affect pathogen survival is by modulating mosquito immune system activity. The presence of midgut bacteria results in basal immune stimulation, and reduction of the adult mosquito midgut microbiota by antibiotic treatment results in decreased production of anti-pathogen immune effector molecules (Jose Luis Ramirez et al. 2012; Dong, Manfredini, and Dimopoulos 2009). This may in turn cause the increase in pathogen susceptibility mentioned above (Dong, Manfredini, and Dimopoulos 2009). Multiple species of bacteria have been shown to cause increased expression of immune system effector genes when present in the midguts of *Anopheles* mosquitoes (Bahia et al. 2014; Jose Luis Ramirez et al. 2012). In fact, the initiation of many anti-*Plasmodium* immune defenses correlate with the

rapid expansion of midgut bacteria in response to increased nutrients from an ingested blood meal (Cirimotich et al. 2010; Pumpuni et al. 1996). Additional evidence that bacteria are capable of indirectly antagonizing a pathogen by activating the mosquito immune system comes from research on PGRPLC, an extracellular PRR upstream of the IMD pathway (Meister et al. 2009). Work by Meister et al. showed that PGRPLC mediated signaling results in the production of anti-microbial effector molecules in response to gut bacteria proliferation after blood feeding (Meister et al. 2009). Knockdown of PGRPLC results in higher *Plasmodium* infection intensities but antibiotic treatment eliminates this effect. This suggests that PGRPLC mediates *Plasmodium* infection in a gut microbe-dependent manner, possibly due to the production of anti-microbial effectors that are active against both bacteria and *Plasmodium* (Cirimotich et al. 2010; Meister et al. 2009; Jose Luis Ramirez et al. 2012). The effectors TEP1, FBN9, and LRRD7 are anti-bacterial and anti-*Plasmodium* effectors, underscoring the importance of bacterial priming of Rel2-mediated anti-*P. falciparum* defense.

A second way the gut microbiota can antagonize *Plasmodium* is through direct interaction with the pathogens themselves. Bacteria are capable of producing a variety of proteins and metabolites that have varying effects on vector competence (Azambuja, Garcia, and Ratcliffe 2005). Recently, multiple species of bacteria with anti-*Plasmodium* activity have

been identified (Cirimotich et al. 2011; Jose Luis Ramirez et al. 2012; Bahia et al. 2014; Jose Luis Ramirez, Short, et al. 2014). Importantly, many of these bacteria not only inhibit pathogen infection in the mosquito, but also cause pathogen killing *in vitro*, suggesting that the bacteria produce anti-pathogen molecules that directly inhibit pathogen survival (Cirimotich et al. 2011; Bahia et al. 2014; Jose Luis Ramirez et al. 2012; Jose Luis Ramirez, Short, et al. 2014). Cirimotich et al. (2011) showed that a strain of *Enterobacter sp.* (*Esp_Z*) isolated from an *Anopheles arabiensis* mosquito in Zambia caused dramatic reductions in *P. falciparum* development when present in *Anopheles gambiae* midguts. This anti-pathogen activity was also evident *in vitro*, suggesting that the bacteria produce molecule(s) with anti-pathogen activity. The authors hypothesized that reactive oxygen species may be one such molecule, and indeed addition of an antioxidant to *Plasmodium* culture reduced the anti-pathogen activity of *Esp_Z*, supporting this hypothesis. Bahia et al. (2014) described multiple species of gut bacteria that had anti-plasmodium activity *in vitro,* including a strain of *Serratia marcescens* that caused very strong pathogen mortality. Anti-pathogen activity of *S. marcescens* was also detectable in the bacterial culture media after removal of the bacteria via filtration, suggesting that *S. marcescens* produces and secretes anti-pathogen molecule(s). Ramirez and Short et al. 2014 reported broad anti-pathogen activity of a *Chromobacterium sp.* (*Csp_P*) isolated from

an *Aedes* mosquito in Panama (Jose Luis Ramirez et al. 2012; Jose Luis Ramirez, Short, et al. 2014). Filtered (*i.e.* bacteria-free) *Csp_P* culture had *in vitro* anti-*Plasmodium* activity against asexual, ookinete and gametocyte stage parasites. Because the anti-*Plasmodium* activity persisted even after *Csp_P* was removed from culture, the authors concluded that pathogen inhibition was likely due to secreted, stable molecule(s) produced by *Csp_P* and released into the media (Jose Luis Ramirez, Short, et al. 2014). These investigations serve to demonstrate that microbes naturally associated with mosquitoes can have powerful anti-*Plasmodium* activity.

In summary, the mosquito bacteriome is a key mediator of anti-*Plasmodium* defense in the mosquito. The IMD pathway is responsible for controlling the midgut bacteria as well as *P. falciparum* infection. Rel2-mediated anti-*Plasmodium* defense can function independently of the bacteriome when Caspar is silenced using RNAi (Garver et al. 2012). However, it is currently unclear if bacteria-independent, anti-*Plasmodium* defenses exist, and if anti-*Plasmodium* defense is just incidental with bacterial priming of the IMD pathway. Furthermore, it is unknown if *Plasmodium* possesses specific PAMPs recognized by the mosquito PRRs which would help clarify the specificity of anti-*Plasmodium* defenses. The bacteriome is also capable of directly antagonizing *Plasmodium* in the mosquito. These findings warrant further investigations into the microbiome

of field-caught mosquitoes in order to discover novel microbes capable of modulating pathogen susceptiblity.

1.8.3 The mosquito mycobiome-fungi

Fungi are eukaryotic organisms with distinctive cell walls composed of chitin that can be found in nearly every ecosystem across the world. Many species are only observed in their asexual form producing non-motile spores called conidia (Osherov 2001). Common fungi include the molds and yeasts, such as the medically important *Aspergillus* genus and the commonly used baker's yeast *Saccarhomyces* (Virginio et al. 2014; Jayaram et al. 2014). Fungi play diverse roles in the ecosystem spanning from commensal to mutualist to pathogenic organisms (George et al. 2013; Underhill and Iliev 2014; Bressano et al. 2010). Fungi are capable of producing a rich array of proteins and secondary metabolites, which allows them to adapt to changing environmental conditions. Some fungi produce toxic secondary metabolites, called mycotoxins, which are extremely resistant to environmental- and temperature-mediated degradation (Marroquín-Cardona et al. 2014). Most mycotoxins are immunosuppressive and mycotoxin-producing fungi are capable of modulating pathogen susceptibility in animals (Marroquín-Cardona et al. 2014; Antonissen et al. 2014). The ability to adapt to diverse

environments allows some fungi to become opportunistic pathogens. For example, fungi belonging to the genus *Aspergillus* are predominantly soil-dwelling saprotrophs, meaning they feed on decaying organic matter, but in the human lung *Aspergillus* can become pathogenic (Warris 2014). Although the term microbiota is primarily used to describe bacteria, fungi are frequently identified in genetic screens of microbiome samples, albeit their numbers tend to be much lower than bacteria (Underhill and Iliev 2014). Still, these fungi are capable of modifying the immune response to pathogens as well as the population size of other microbiota (Underhill and Iliev 2014; Chiapello et al. 2004). Recently, there has been renewed interest in the role of fungi in the microbiome of humans and other organisms such as mosquitoes (Underhill and Iliev 2014; Ricci, Mosca, et al. 2011; Cappelli et al. 2014). These endogenous fungi are collectively referred to as the mycobiome.

There are far fewer peer-reviewed articles exploring the mosquito mycobiome as opposed to the bacterial microbiome, and the effects of fungi on pathogen susceptibility in mosquitoes are largely unknown. Most of the research addressing mosquito-fungi interactions is descriptive or focuses on the use of pathogenic fungi or toxic fungal products to control mosquito populations (Maketon, Amnuaykanjanasin, and Kaysorngup 2014; Fang et al. 2011; da Costa and de Oliveira 1998; Geris et al. 2008). Much like bacteria, mosquitoes are exposed to fungi as larvae in the water, and as adults either

by ingesting fungi in sugar meals or by external physical contact with conidia (da Costa and de Oliveira 1998; da S Pereira et al. 2009; Tajedin et al. 2009; Lynch et al. 2012). There is evidence to suggest that *Penicillium* molds and other filamentous fungi dominate the mosquito mycobiome without any apparent detriment to the health of the mosquito (da S Pereira et al. 2009; da Costa and de Oliveira 1998). In addition to filamentous fungi, many yeasts have been isolated from mosquitoes in both the lab and field (Frants and Mertvetsova 1986; Cappelli et al. 2014; Ricci, Mosca, et al. 2011; Ricci, Damiani, et al. 2011; Gusmão et al. 2010). Yeasts belonging to the genera *Candida*, *Pichia*, and *Wickerhamomyces* have been identified in *Aedes* and *Anopheles* mosquitoes in both the lab and field (Gusmão et al. 2010; Ricci, Damiani, et al. 2011; Frants and Mertvetsova 1986). In nature, yeasts are typically found in sugar-rich environments such as plant nectars, which may be the source of acquisition for mosquitoes (Ricci, Mosca, et al. 2011; Ricci, Damiani, et al. 2011). Other filamentous fungi, such as some species of *Aspergillus* and *Penicillium* are pathogenic (Maketon, Amnuaykanjanasin, and Kaysorngup 2014; Mohanty and Prakash 2010). A number of true entomopathogenic fungi parasitize mosquitoes, including fungi that belong to the generas *Beauveria* and *Metarhizium* (Scholte et al. 2004). These pathogens are capable of reducing mosquito lifespan as well as vector

competence for pathogens (Garza-Hernández et al. 2013; Dong, Morton, et al. 2012).

1.8.4 Fungi in mosquito immunity and pathogen susceptibility

Tripartite interactions between the mosquito immune system, *Plasmodium*, and fungi have not been studied in depth. *Drosophila* has served as a guide for dissecting mosquito immunity to fungi. Studies have shown that the Toll and JAK-STAT pathways are the primary mediators of anti-fungal defense in the mosquito (Cirimotich et al. 2010). Melanization is also an important anti-fungal defense against entomopathogenic fungi (Yassine, Kamareddine, and Osta 2012). However, these studies have almost exclusively used entomopathogenic fungi to probe immune defenses. Many fungi do not appear to be pathogenic to mosquitoes, including *Penicillium* molds identified in the mosquito midgut. The IMD pathway mediates much of the immune response in the mosquito midgut, but it does not appear to play a substantial role in anti-fungal defense. More research is necessary to characterize anti-fungal defenses in the midgut. *B. bassiana* infections of *Aedes* mosquitoes are capable of priming anti-viral defenses mediated by the JAK-STAT pathway, but fungi have not been probed for their ability to prime anti-*Plasmodium* defenses in *Anopheles*. The location of filamentous fungi in

the midgut places them in close proximity to invading *Plasmodium* parasites. Unlike bacteria, however, fungi have not been explored for their ability to produce anti-*Plasmodium* effectors *in vivo*. It is clear that fungi are naturally associated with mosquitoes, and further investigation into their potential for modulating mosquito-*Plasmodium* interactions is warranted.

1.9 Summary and justification of thesis research

In the first part of the following work, I investigate the specificity of anti-*P. falciparum* defense in *A. gambiae*. Mosquito immune responses to *P. falciparum* are dominated by IMD pathway anti-*Plasmodium* defenses. The midgut microbiota are implicated in activating the IMD pathway, and the most potent effector molecules are only modestly regulated upon *P. falciparum* infection. Furthermore, research has failed to identify PAMPs associated with *P. falciparum*, which suggests that anti-*Plasmodium* defense could be bacteria-dependent. In this work, I analyze the global transcriptome of *A. gambiae* infected with *P. falciparum* for regulated transcripts in the presence or absence of the endogenous microbiota. I select and then use a variety of techniques to characterize two genes involved in what appears to be a *Plasmodium*-specific defense response. These unique findings

demonstrate the existence of IMD and bacteria-independent anti-*P. falicparum* defenses.

In the second part of this work, I explore the ability of fungi to modulate *Plasmodium* susceptibility in *Anopheles* mosquitoes. Although bacteria have been heavily investigated for their ability to modulate mosquito immune defenses and in turn *Plasmodium* infection, there has been a significant lack of attention given to fungi. Like bacteria, fungi are in intimate contact with mosquitoes throughout their lives and are capable of producing a plethora of compounds with potential anti-pathogen activity. However, only entomopathogenic fungi have been researched in depth. In this work, I isolate fungi from field-caught mosquito midguts and identify a species of *Penicillium*. Surprisingly, I discover that this species of fungus can modulate increased susceptibility to *Plasmodium* infection in *Anopheles*. To better understand this novel finding, I use a variety of experiments to narrow down the mechanism underlying *Penicillium*-enhanced *Plasmodium* infection in *Anopheles*.

1.9.1 Specific Aim 1 **(Chapter 2)**: To characterize bacteria-independent immune defenses against *Plasmodium falciparum* in *Anopheles gambiae*.

1.9.2 Specific Aim 2 **(Chapter 3):** To characterize tripartite interactions between the *Anopheles* immune system, *Plasmodium* parasites, and a filamentous fungus belonging to the genus *Penicillium*.

Chapter 2: Bacteria- and IMD Pathway- Independent Immune Defenses Against *Plasmodium falciparum* in *Anopheles gambiae* (Adapted from Blumberg *et al.* 2013)

2.1 Abstract

The mosquito *Anopheles gambiae* uses its innate immune system to control bacterial and *Plasmodium* infection of its midgut tissue. The activation of potent IMD pathway-mediated anti-*Plasmodium falciparum* defenses is dependent on the presence of the midgut microbiota, which activate this defense system upon parasite infection through a peptidoglycan recognition protein, PGRPLC. Transcriptomic and reverse genetic analyses were employed to compare the *P. falciparum* infection-responsive transcriptomes of septic and aseptic mosquitoes and to determine whether bacteria-independent anti-*Plasmodium* defenses exist. Antibiotic treated aseptic mosquitoes mounted molecular immune responses representing a variety of immune functions upon *P. falciparum* infection. Among other immune factors, the analysis uncovered a serine protease inhibitor (SRPN7) and Clip-domain serine protease (CLIPC2) that were transcriptionally induced in the midgut upon *P. falciparum* infection, independent of bacteria.

It was also shown that SRPN7 negatively and CLIPC2 positively regulate the anti-*Plasmodium* defense, independently of the midgut-associated bacteria. Co-silencing assays suggested that these two genes may function together in a signaling cascade. Neither gene was regulated, nor modulated, by infection with the rodent malaria parasite *Plasmodium berghei*, suggesting that SRPN7 and CLIPC2 are components of a defense system with preferential activity towards *P. falciparum*. Further analysis using RNA interference determined that these genes do not regulate the anti-*Plasmodium* defense mediated by the IMD pathway, and both factors act as agonists of the endogenous midgut microbiota, further demonstrating the lack of functional relatedness between these genes and the bacteria-dependent activation of the IMD pathway. This is the first study confirming the existence of a bacteria-independent, anti-*P. falciparum* defense. Further exploration of this anti-*Plasmodium* defense will help clarify determinants of immune specificity in the mosquito, and expose potential gene and/or protein targets for malaria intervention strategies based on targeting the parasite in the mosquito vector.

2.2 Rational and Hypothesis

Recent studies have shown a dependence on bacteria-mediated activation of the IMD pathway to launch an effective anti-*Plasmodium* immune response in the mosquito gut tissue, which harbors a variety of mostly Gram-negative bacteria (Meister et al. 2009). The IMD pathway contributes to microbial homeostasis in this tissue by maintaining a continuous basal level activity, which is stimulated by the midgut microbiota (Dong, Manfredini, and Dimopoulos 2009). PGRPLC has been shown to play a role in this basal level immune activation by sensing bacteria in the gut tissue (Meister et al. 2009; Dong, Manfredini, and Dimopoulos 2009). Interestingly, PGRPLC has also been shown to be essential for triggering an anti-*P. falciparum* response through the IMD pathway, but only when bacteria are present in the gut tissue (Meister et al. 2009). RNAi-mediated depletion of PGRPLC from antibiotic-treated *A. gambiae,* which have a greatly reduced microbial flora, does not influence the mosquito's susceptibility to the parasite, as it does in non-antibiotic-treated septic mosquitoes, suggesting that PGRPLC–mediated activation of the IMD pathway's anti-*Plasmodium* defense depends on the presence of midgut bacteria (Dong, Manfredini, and Dimopoulos 2009; Meister et al. 2009). We and others have previously shown that the anti-*Plasmodium* effectors FBN9, LRRD7*,* and TEP1 are also involved in controlling bacterial proliferation in the midgut tissue, corroborating the intimate relationship between anti-

bacterial and anti-*Plasmodium* defenses (Dong et al. 2006; Dong, Manfredini, and Dimopoulos 2009). Here we wanted to investigate whether *P. falciparum* ookinete infection of the mosquito midgut activates anti-parasitic immune responses in a bacteria-independent manner. Arrighi *et al.* previously investigated the role of *Plasmodium* glycosylphosphatidylinositol (GPI) anchors in the induction of an immune response, and although their study documented the induction of some immune genes, the potential anti-*Plasmodium* action and possible dependence and relationship of these genes to the microbiota were not investigated (Arrighi et al. 2009).

Since the transcriptome of an organism, tissue, or cell type represents a reflection of a physiological state such as immune response, we used whole-genome microarray analysis to investigate the *P. falciparum* infection-responsive transcriptome in septic and aseptic mosquitoes in order to identify and characterize bacteria-independent immune response signatures and factors. Our analysis revealed a variety of putative immune genes that are regulated upon *Plasmodium* infection in the absence of midgut microbiota, and we specifically focused on a clip-domain serine protease (CLIPC2) and a serine protease inhibitor (SRPN7), showing that these genes modulate the intensity of the *P. falciparum* infection in the absence of bacteria. CLIPC2 also controlled systemic bacterial infection and both genes modulated the proliferation of the midgut microflora, indicating their functional versatility.

Interestingly, our study suggests that CLIPC2 and SRPN7 may be part of the same protease signaling cascade and that their anti-*P. falciparum* function is independent of the IMD pathway, since these factors were not regulated by and did not regulate this pathway. Our study points to the existence of bacteria-independent anti-*Plasmodium* defenses, possibly relating to as-yet unknown immune pathways and mechanisms.

2.3 Materials and Methods

2.3.1 Mosquito Rearing, RNA Isolation, and cDNA Synthesis

A. gambiae Keele strain mosquitoes were maintained on a 10% sucrose solution with 12-h light/dark cycles at 27°C and 80% humidity (Hurd et al. 2005; Crampton, Beard, and Louis 1997). At specific time points, mosquitoes were anesthetized on ice, and either whole mosquitoes or specific tissues were dissected and collected. RNA was extracted from tissues using the RNeasy kit (Qiagen), and RNA yields were quantified using the Nanodrop 2000 (ThermoFisher) following treatment with DNase (Ambion) cDNA was synthesized from extracted RNA using the Oligo-DT primer and M-MLV Reverse Transcriptase (Promega).

2.3.2 Primer Design and qRT-PCR

The Primer 3 Program (http://frodo.wi.mit.edu) was used to design all primers, except for the bacterial *16s* primers (Nadkarni et al. 2002). Real-time quantitative PCR (qRT-PCR) to assess transcript abundance and silencing efficiency was performed as described in (Dong et al. 2006). Transcript abundance was quantified with Sybr Green PCR Master Mix (Applied Biosystems) using the ABI StepOnePlus Real-Time PCR System and ABI StepOne Software. PCR reactions were performed in duplicate, and melting curve analysis was used to analyze primer specificity. Transcript abundance of target genes were first normalized to the within sample transcript abundance of the mosquito ribosomal *S7* gene, and fold changes between samples were determined using the ΔΔct method.

2.3.3 RNAi Gene Silencing

The HiScribe T7 *in vitro* Transcription Kit (New England Biolabs) was used to generate double-stranded RNA (dsRNA) from PCR-amplified gene oligos.Gene silencing was performed essentially as described in (Dong et al. 2006; Stéphanie Blandin et al. 2002). In brief, 3- to-4-day-old female *A. gambiae* were cold-anesthetized, and cohorts were injected with control *GFP*

dsRNA or experimental gene-specific dsRNA at a concentration of 3μg/μL (207ng dsRNA per mosquito). Pools of 15 mosquitoes were collected 1-4 days post-dsRNA injection, and silencing efficiency was assessed by qRT-PCR.

2.3.4 Quantification of Endogenous Mosquito Midgut Bacteria

Colony forming units (CFU) from mosquito midguts were quantified in control untreated, antibiotic-treated, and gene-silenced mosquitoes as described (Dong, Taylor, and Dimopoulos 2006; Dong, Manfredini, and Dimopoulos 2009). For the gene silencing experiment , 3-4 day old female *A. gambiae* mosquitoes were injected with dsRNA as described in the section "RNAi Gene Silencing." 3 days post-injection of dsRNA, mosquito midguts were dissected and processed as described below. At the indicated time points, female mosquitoes were collected, surface-sterilized in ethanol, and washed with 1x PBS, and their midguts were dissected in sterilized 1x PBS. Collected midguts were homogenized, and serial dilutions of homogenate were plated on LB agar plates. After incubation for 2-3 days at 27^{o}C under aerobic or anaerobic conditions, the CFUs per plate were counted, and a titer of CFU/midgut was calculated.

2.3.5 Antibiotic Treatment

For antibiotic treatment, adult female mosquitoes were collected post-eclosion and given a sterile 10% sucrose solution containing 75 µg/mL gentamicin sulfate (Quality Biological) and 100 units (µg)/mL of penicillin-streptomycin (Invitrogen). Treatment was carried out for at least 3 days, and antibiotic-containing sucrose was changed daily to ensure adequate elimination of bacteria. To validate the efficiency of antibiotic treatment, midguts from control untreated and experimental antibiotic treated mosquitoes were subjected to culture-dependent CFU and culture-independent enumeration assays. The culture-dependent CFU assay tested sugar-fed, antibiotic-treated sugar-fed, blood-fed, and antibiotic-treated blood-fed mosquito midguts under aerobic or anaerobic conditions: Individual midguts were collected from sugar-fed and blood-fed (24 h post-blood feeding) adult females essentially as described above. Individual midgut samples were homogenized in 1x PBS, and serial dilutions of the homogenate were spread on LB agar plates cultured under aerobic or anaerobic conditions. Anaerobic conditions were achieved using the BD GasPak EZ Anaerobe Container System (BD). The culture-independent assay involved qRT-PCR of the bacterial 16s ribosomal gene from the two sugar-fed and two blood-fed groups listed above.

2.3.5 Plasmodium Challenge

P. falciparum and *P. berghei* challenges were accomplished following a standard protocol (Dong et al. 2006). For *P. falciparum* infection: Three days post-dsRNA injection, mosquitoes fed on NF54W strain gametocytes in human blood through a membrane feeder at 37°C. Unfed mosquitoes were removed within the first day post-infection, and engorged mosquitoes were maintained at 27°C for up to 8 days. For *P. berghei* infection: Three days post-dsRNA injection, mosquitoes were allowed to feed on Swiss Webster mice infected with the WT Anka 2.34 strain of the parasite. Unfed mosquitoes were removed within the first day post-infection, and engorged mosquitoes were maintained at 19°C for 14 days. *P. falciparum*- and *P. berghei*-infected mosquito midguts were dissected and stained with 0.1% mercurochrome, and oocyst numbers were counted using a light microscope (Olympus).

2.3.6 Microarray Hybridization and Data Analysis

All probe sequences, probe preparation, microarray construction, and microarray hybridizations were performed essentially as previously described (Dong et al. 2006). Control (Cy3-labeled) and experimental (Cy5-labeled) cRNA probes were generated from 2-3 µg of RNA according to the

manufacturer's instructions (Agilent Technologies Low RNA Input Linear Amplification Kit). Probe hybridization to the microarray slides was accomplished using 2 µg of cRNA, and microarray slides were washed and dried 16 h post-hybridization. Slides were scanned using an Axon GenePix 4200AL scanner at 10-µm pixel size (Axon Instruments, Union City, California, USA); 60% laser power was used, and the photomultiplier tube (PMT) voltage was adjusted to maximize the dynamic range and minimize pixel saturation. GenePix software was used to analyze the scanned images. Cy5 and Cy3 values were processed and subjected to statistical analysis using the TIGR, MIDAS, and TMEV software packages (Dudoit, Gentleman, and Quackenbush 2003). The minimum signal intensity accepted was 100 fluorescent units, and a signal-to-background cutoff ratio of 2.0 was used. Three biological replicates and a pseudo replicate were performed for each group. Median fluorescent values for good spots were normalized by the LOWESS normalization method (Yang et al. 2002). Statistical analysis of Cy5/Cy3 ratios was performed using a *t*-test with significance at $p<0.05$, and the cutoff value for significant gene regulation was 0.75 on a log2 scale (Yang et al. 2002). The microarray data was assembled in the Minimum Information About a Microarray Experiment (MIAME)-compliant format and is available in the public Gene Expression Omnibus (GEO) database under accession GSE49690.

2.3.7 Bacterial Challenge

Mosquitoes were injected with control ds*GFP* or experimental dsRNA constructs. Three days post-injection, mosquitoes were cold-anesthetized and injected with 69 nL of either Gram-positive *Staphylococcus aureus* or Gram-negative *Escherichia coli*, at the optical density mentioned at the end of this section. Glycerol stocks of both species of bacteria were used to inoculate LB broth cultures, which were grown in a shaker overnight at 37^{o} C for approximately 18 h. The cultures were centrifuged to a pellet that was then washed with 1x PBS three times. A biophotometer (Eppendorf) was used to measure optical density (OD600), and samples were diluted with 1x PBS to the appropriate absorbance prior to injection (OD600; *S. aureus* = 0.35, *E. coli* = 3.0).

2.3.8 Statistical Analysis

The Graphpad Prism 5 (Graphpad Prism®) software package was used to perform statistical analyses. The particular test used is indicated in the captions of each respective figure.

2.4 The midgut microbiota are removed by antibiotic treatment

To examine the impact of *P. falciparum* infection on the mosquito midgut and carcass transcriptomes in the presence or absence of midgut bacteria, we used *A. gambiae* whole genome microarrays to compare the mRNA abundance of *P. falciparum*-infected and -naïve mosquitoes of antibiotic- and non-antibiotic treated cohorts. Depletion of the great majority of midgut bacteria was achieved by treating mosquitoes with a broad-spectrum antibiotic cocktail containing 75 ug/ml gentamycin, 100 units/ml penicillin and 100 ug/ml streptomycin for 4 days through their sugar meal, prior to feeding on *P. falciparum* gametocytes. To assess the efficacy of the antibiotic treatment in the removal of the midgut microbiota, we assayed colony forming unit (CFU) growth on LB agar of both the aerobic and anaerobic bacteria present in sugar-fed and 24-h blood-fed mosquito midguts (Figure 2.1 A,B). Although culturing bacterial isolates exclusively on LB agar may limit the ability to capture the entire spectrum of bacterial species present in the mosquito midgut, we have observed near identical growth of the same bacteria on a variety of mediums (LB, Yeast extract-peptone dextrose, blood agar), (Dimopoulos lab, unpublished data). Our assays showed that no CFU could be detected in antibiotic-treated mosquitoes. Since some midgut bacteria may be unculturable we also we determined the relative microbial

load of these samples using qRT-PCR with universal primers amplifying the bacterial 16s ribosomal RNA (16s rRNA), (Table 2.1). The 16s rRNA was amplified 63-fold higher in septic sugar-fed and 272-fold higher in septic blood-fed midguts when normalized to 16s rRNA from aseptic sugar-fed and aseptic blood-fed midguts, respectively (Figure 2.1 C, D). This PCR-amplified bacterial nucleic acid could represent the remains of dead bacteria that existed in the midguts prior to the antibiotic treatment, and the large difference in 16s rRNA abundance between septic and aseptic midguts suggests near complete removal of the microbiota by antibiotic treatment. It is possible that RNA was amplified from a small number of bacteria unaffected by antibiotic treatment. Nevertheless, we have previously demonstrated that the removal of bacteria through antibiotic treatment is efficient to impact on the *Plasmodium* infection phenotype, and for practical purposes we consider our antibiotic-treated mosquitoes aseptic when compared non-antibiotic treated septic mosquitoes (Dong, Manfredini, and Dimopoulos 2009).

Figure 2.1 Removal of bacteria from the midgut by antibiotic treatment of adult females

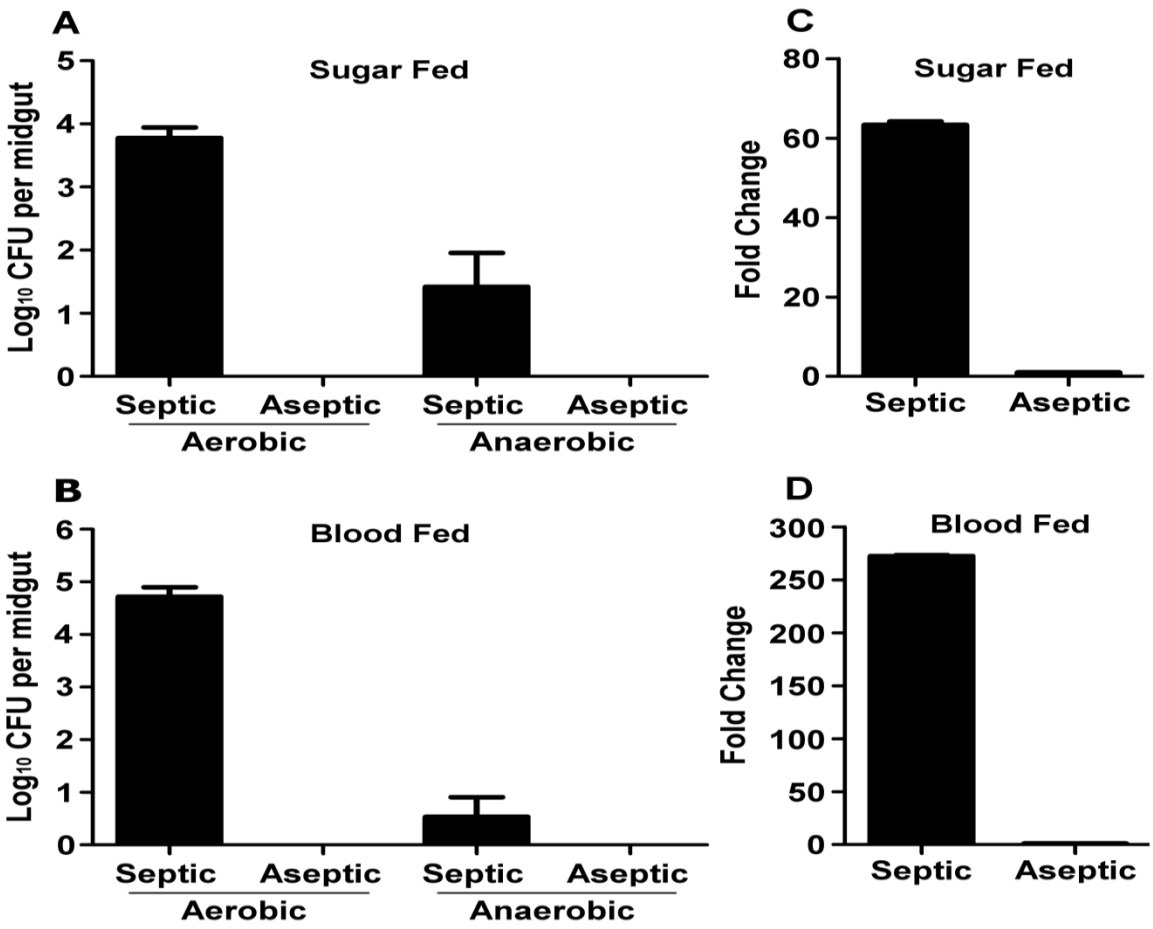

Figure 2.1.Removal of bacteria from the midgut by antibiotic treatment of adult female mosquitoes. Culture-dependent methods of bacterial cultivation (aerobic vs. anaerobic conditions) were unsuccessful at growing any bacteria from the midguts of aseptic (antibiotic-treated) mosquitoes after feeding on either (A) sugar or (B) 24h post-blood meal. In contrast, bacteria from the midguts of septic (untreated) mosquitoes fed on (A) sugar or (B) 24h post-blood meal could be cultured under aerobic and anaerobic conditions. (C) and (D) Culture-independent analysis of bacterial 16s rRNA by qRT-PCR measured almost no 16s rRNA in aseptic mosquitoes (sugar or blood-fed). For (A) and (B), colony forming units (CFU) from three biological replicates were pooled. For (C) and (D), 10 midguts from each treatment were assessed individually by qRT-PCR, and the relative amount of 16s rRNA from aseptic midguts (sugar or blood-fed) was compared to that of the septic (sugar or blood-fed) midgut groups, respectively. Black bars represent the mean CFU or mean -fold change, and error bars represent the standard error of the mean.

Table 2.1 Primers Used

Gene Target	AGAPID	Primer Name	Primer Sequence	Primer Use
GFP	n/a	rGFP-Forward	TAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGAGCTGT	RNAi
		rGFP-Reverse	TAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCATGCCG	
S7	AGAP010592	S7-Forward	CCATCCTGGAGGATCTGGTA	qRT-PCR
		S7-Reverse	GATGGTGGTCTGCTGGTTCT	
Serpin7	AGAP007693	rSRPN7-Forward	TAATACGACTCACTATAGGGTATCGCAGGAGCTGGAAGAT	RNAi
		rSRPN7-Reverse	TAATACGACTCACTATAGGGGTTTGCTCCATCGCAAACTT	
		SRPN7-F	TGAAGCAAAGCACCTTTGTG	qRT-PCR
		SRPN7-R	GTATTGCGGACACGGAATTT	
ClipC2	AGAP004317	rCLIPC2-Forward	TAATACGACTCACTATAGGGTTAGTGCATGTAGCGCGAAC	RNAi
		rCLIPC2-Reverse	TAATACGACTCACTATAGGGGGGACGTGTAGCCACAGATT	
		CLIPC2-Forward	GATCAGCTCGGAAGATGAGG	qRT-PCR
		CLIPC2-Reverse	CTCCGACACACGCGTATAGA	
Tep1	AGAP010815	TEP1-Forward	TCCAGCGTATGTGGTTGTGT	qRT-PCR
		TEP1-Reverse	TCGCACAAATTCTGCTTGTC	
Fbn9	AGAP011197	FBN9-Forward	TTGTGATGAAGGAGCACAGC	qRT-PCR
		FBN9-Reverse	GCTTGATCCAACCGACTGAT	
Lrrd7	AGAP005693	LRRD7-Forward	TCGGTGAGCAACAGTTTGAC	qRT-PCR
		LRRD7-Reverse	CAGGTCGAGATGGGTGAACT	
16s	n/a	16s-Forward	TCCTACGGGAGGCAGCAGT	qRT-PCR
		16s-Reverse	GGACTACCAGGGTATCTAATCCTGTT	

Table 2.1 Primers used in this study. PCR primer sequences used in this study. "Gene Target" displays the target gene of the corresponding primer. "AGAPID" lists the Vectorbase identifier if applicable such as in the case of a primer designed to produce double-stranded RNA for targeted gene silencing. "Primer name" is an arbitrary identifier for a particular primer sequence. "Primer sequence" displays the forward and reverse primers designed for a specific gene. "Primer use" shows the use of a given primer (i.e. RNAi is for generating double stranded RNA whereas qRT-PCR indicates a primer was used in real-time PCR analyses).

2.5 Transcriptome responses to Plasmodium infection

We then compared the genome-wide transcript abundance between infected and non-infected mosquitoes of septic and aseptic cohort sat 24 h post-ingestion of a *P. falciparum* gametocyte culture or naïve blood; a time period when ookinetes invade the midgut epithelium and the microbial flora has expanded by a 10- to 20-fold in the nutrient-rich blood (Figure 2.2) (Han et al. 2000; Pumpuni et al. 1996). Regulation was determined by assessing Log2 ratio values of transcript abundance that were above our cutoff for significance of 0.75, or below -0.75. *P. falciparum* infection induced changes in the abundance of as many as 2,183 and 2,429 transcripts in whole mosquitoes belonging to a variety of functional groups in aseptic and septic mosquitoes, respectively, representing approximately 16% and 18% of the *A. gambiae* transcriptome (Figure 2.2 A, B). The abundance of 1,556 transcripts was regulated in the aseptic midguts, as compared to 1,760 in the septic midguts, and 1,154 and 916 transcripts displayed changes in the aseptic and septic carcasses, respectively, upon *P. falciparum* infection (Figure 2.2 C). The abundance of 458 transcripts changed (204 induced and 254 repressed) in the same direction in both aseptic and septic midguts, suggesting that *P. falciparum* infection, and not the presence or absence of the midgut bacteria, was likely to account for this response (Figure 2.2 C). In comparison, the

transcript abundance of only 96 genes was similarly regulated (50 induced and 46 repressed) in both the aseptic and septic carcasses, suggesting that *Plasmodium* ookinete invasion and traversal of the midgut has a greater impact on this tissue than on the rest of the mosquito (Figure 2.2 C). The abundance of only 48 transcripts displayed an opposite pattern of change between the aseptic and septic midguts (39 induced in aseptic midguts, 9 repressed in aseptic midguts), suggesting that the presence or absence of midgut bacteria influences the expression of these particular genes after *Plasmodium* infection (Figure 2.2 C). In addition, 46 transcripts with predicted immune functions were uniquely regulated in the septic midgut (11 induced and 35 repressed), suggesting that tripartite interactions between *Plasmodium* parasites, midgut bacteria, and the midgut epithelium affect the expression of this set of genes (Table 2.2, see supplementary file).When we compared transcript abundance between the midgut and carcass within the aseptic group (Figure 2.2 C), we observed 486 regulated genes (401 induced and 85 repressed) shared between these tissue compartments. This is over double the number of regulated transcripts (66 induced and 133 repressed) that were shared between the midgut and carcass within the septic group. This observation suggests that the presence of midgut bacteria accounts for larger differences between the midgut and carcass transcriptomes, whereas

in the absence of midgut bacteria there is more similarity in the transcriptome between the two tissue compartments.

Transcripts of a suppressor of cytokine signaling (SOCS, AGAP001623, Log2 = 0.97) were upregulated in the *P.falciparum* infected septic midguts, as was a SOCS negative regulator of the *A. gambiae* JAK-STAT pathway that had been previously implicated as a host factor in *Plasmodium* infection (Gupta et al. 2009). The transcripts of the secreted modular serine protease 22D (SCRASP1, AGAP005625, Log2 = 0.88) were also upregulated in the septic midguts. SCRASP1 has a chitin-binding domain that has been hypothesized to sense chitin in response to injury and to transduce signals via the serine protease domain, even though the signaling pathway to which SCRASP1 belongs has remained elusive (McTaggart et al. 2009; Danielli et al. 2000). Also upregulated in the septic parasite-infected midguts were transcripts of spaetzle-like cytokine 2 (SPZ2*,* AGAP006483, Log2 = 0.75),which may be involved in TOLL pathway activation (I. Akhouayri et al. 2011). Transcripts of the thioester-containing protein 1 (TEP1, AGAP010815, Log2 = 0.72), an IMD-pathway associated effector molecule with strong anti-*Plasmodium* activity, was close to being significantly regulated in accordance with previous observations of *P. falciparum* infection in the septic gut (Garver, Dong, and Dimopoulos 2009; Dong et al. 2006).

Ultimately, we were interested in identifying the genes involved in bacteria-independent anti-*Plasmodium* responses, and therefore we focused on transcripts displaying increased abundance in the parasite-infected aseptic midguts, placing a particular emphasis on those with predicted immune functions. Of the 783 transcripts specifically enriched in the aseptic midguts, 17 had predicted functions in immunity, whereas the majority of genes of this group belonged to other diverse or unknown functional groups. Two genes that displayed changes in their transcript abundance in *P. falciparum*-infected aseptic midguts, LRRD1 (AGAP000360, Log2 = 1.14) and LRRD18 (AGAP000054, Log2 = 1.3), belong to the leucine rich repeat domains (LRRD) gene family, which also contains members with a putative function in pattern recognition and to play key roles in anti-*Plasmodium* functions (Dong et al. 2006; Osta, Christophides, and Kafatos 2004; Riehle et al. 2006). Fibrinogen-related proteins have been implicated in the pattern recognition processes of human and rodent malaria parasites, and two FBN genes (FBN34 AGAP001554, Log2 = 1.04) and a novel gene, (XM_001231172, AGAP010772, Log2 = 1.86) encoding such putative immune factors were upregulated in the aseptic midguts by parasite infection (Dong and Dimopoulos 2009). Another upregulated gene, SCRB5 (AGAP002738, Log2 = 3.17), belongs to a class of scavenger receptors with diverse roles in pattern recognition, phagocytosis, and *Plasmodium* infection (González-Lázaro et al.

2014; Chung and Kocks 2011; Nehme et al. 2011). A non-alternatively spliced region of the AGDSCAM gene (AGAP007092, Log2 = 1.49) was also upregulated. Transcripts of this gene, in theory, can produce over 31,000 splice forms through alternative splicing, and AGDSCAM already has a recognized role in pattern recognition and immunity to *Plasmodium* infection (Dong, Taylor, and Dimopoulos 2006). Also upregulated in the aseptic midguts were a number of serine proteases and serine protease inhibitors. Studies have previously described roles for these gene families in melanization, immune pathway activation, and anti-parasitic activity (Abraham et al. 2005; Michel et al. 2005; Fullaondo et al. 2011).

Because of the central role of serine protease cascades in regulating insect immune defenses, we focused the remainder of our analysis on a clip-domain serine protease C2 (CLIPC2, AGAP004317, Log2 = 0.96) and a serine protease inhibitor 7 (SRPN7, AGAP007693, Log2 = 4.16) that were specifically upregulated in the parasite-infected, aseptic mosquito midgut (An, Jiang, and Kanost 2010; Volz et al. 2006). Their regulation by *P. falciparum* infection in the absence of the midgut microbiota suggested that they were likely to be involved in regulating bacteria-independent anti-*Plasmodium* defenses. Serpins represent a large family of negative regulators of proteolytic cascades that play a critical rolesin a variety of processes both vertebrates and invertebrates (Gettins 2002). In humans, serpins regulate

finely tuned processes such as fibrinolytic cascades, clotting, and inflammatory reactions (Stein and Carrell 1995). In arthropods, serpins have been shown to regulate components of the prophenoloxidase (PPO) pathway, which is responsible for the melanization of pathogens, as well as to inhibit processes upstream of the seminal TOLL pathway, which functions in both development and innate immunity (Fullaondo et al. 2011; Volz et al. 2006; Morisato and Anderson 1995; Ligoxygakis, Roth, and Reichhart 2003). *A. gambiae* SRPN7 has 1:1 orthologs in both the yellow fever mosquito *Aedes aegypti* and the Southern house mosquito *Culex quinquefasciatus*, suggesting that the gene has a conserved, mosquito-specific function. Clip-Domain serine proteases also belong to a large gene family, but unlike the serine protease inhibitors (serpins), they are only found in arthropods (Christophides et al. 2002; Ross et al. 2003). Functional studies have demonstrated a role for Clip proteases in the activation of prophenoloxidases (PPOs), which mediate melanization defenses as well as the TOLL pathway (Volz et al. 2006; Jang et al. 2006; Kambris et al. 2006; Kanost, Jiang, and Yu 2004; Tang et al. 2006). In mosquitoes, there are five sub-families of Clip proteases (A, B, C, D, and E), and studies on subfamily A and B members have shown that some of these genes regulate the PPO pathway (Volz et al. 2006; Volz et al. 2005; Paskewitz, Andreev, and Shi 2006; An et al. 2011). Although little is known about the role of subfamily C members in mosquitoes, it is worth noting that

in *Drosophila* subfamily C members include SNAKE and PERSEPHONE, which are involved in TOLL pathway activation in the context of development and immunity, respectively (Ligoxygakis et al. 2002; DeLotto and Spierer 1986). The catalytic triad (His, Asp, Ser) is present in this clip protease indicating likely enzymatic activity similar to what was observed in another clip serine protease (Kellenberger et al. 2011). Like SRPN7, CLIPC2 has 1:1 orthologs in both *C. quinquefasciatus* and *A. aegypti,* again suggesting a mosquito-specific gene function.

Figure 2.2 **Global gene regulation of mosquitoes at 24 h post-*P. falciparum* infection under septic and aseptic conditions.**

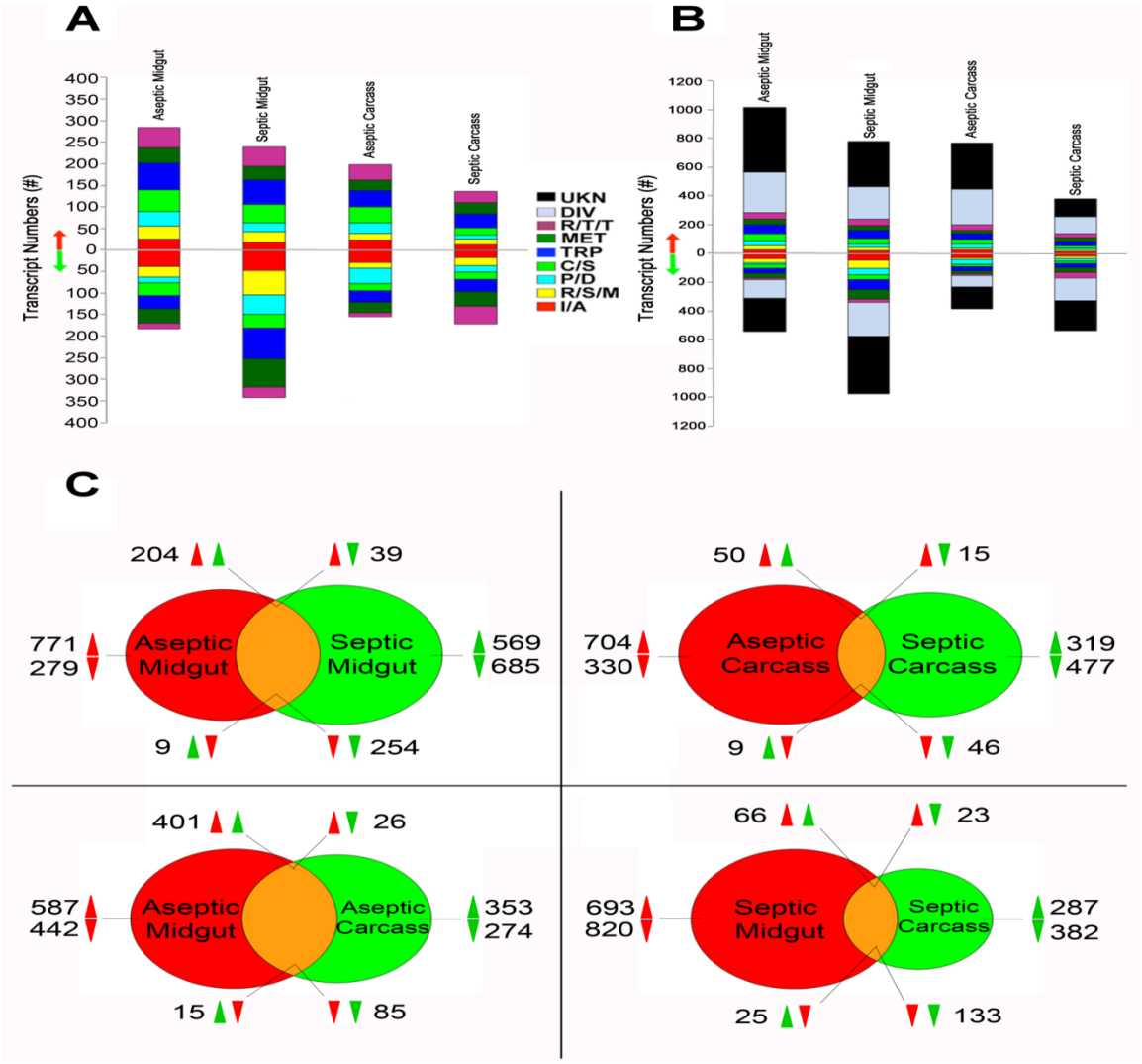

Figure 2.2: Global gene regulation of mosquitoes at 24 h post-P. falciparum infection under septic and aseptic conditions. (A) Numbers of up- or down-regulated genes in distinct functional groups according to tissue (midgut/carcass) and treatment (septic/aseptic) at 24 h post-*P. falciparum* infection (not including DIV/UKN). (B) Same as in (A) but including DIV/UKN. (C) Venn diagrams comparing the total numbers of regulated genes between tissues and treatments. Red arrows correspond to the tissues/treatments in the left circles, and green arrows correspond to the tissues/treatments in the right circles. The arrow direction indicates up- or down-regulation. I/A: putative immunity and apoptosis; R/S/M: oxidoreductive, stress-related and mitochondrial; C/S: cytoskeletal, structural; MET: metabolism; R/T/T: replication, transcription, translation; P/D: proteolysis, digestion; TRP: transport; DIV: diverse; UKN: unknown functions.

2.6 Infection-responsiveness of SRPN7 and CLIPC2

Quantitative real-time PCR (qRT-PCR) assays were used to confirm the up-regulation of SRPN7 and CLIPC2 in aseptic *P. falciparum*-infected mosquitoes (Table 2.2). The infection-responsive increase in SRPN7 transcript abundance was greatest in the aseptic midgut, although it was modest when compared to that of mosquitoes fed on naïve blood (Figure 2.3 A). Since SRPN7 transcripts were previously detected at low levels in adult mosquitoes, the increase in transcript abundance upon *Plasmodium*-infection of the aseptic midgut is intriguing (Suwanchaichinda and Kanost 2009). SRPN7 transcripts have previously been reported to be upregulated in the midguts of mosquitoes fed on a blood meal mixed with Gram-positive and Gram-negative bacteria (Dong, Manfredini, and Dimopoulos 2009). Analysis of CLIPC2 has shown nearly a 5-fold increase in transcript abundance after *P. falciparum* infection of aseptic mosquito guts at 24 h after feeding on a gametocyte culture, when compared to mosquitoes fed on naive blood (Figure 2.3 B). Our earlier studies on the IMD pathway-regulated mosquito transcriptome have suggested that SRPN7 and CLIPC2 are not regulated by the IMD pathway (Garver, Dong, and Dimopoulos 2009). It is possible that differences in *P. falciparum* infection intensity and/or prevalence between septic and aseptic mosquitoes could have influenced the transcript abundance

of these genes, but it is technically challenging to normalize the amount of pre-invasive parasites between antibiotic-treated and untreated cohorts. Nevertheless, our data prove bacteria-independent induction of SRPN7 and CLIPC2 upon *P. falciparum* infection. Overall, the pattern of gene transcript regulation detected by qRT-PCR supported our microarray-based studies, confirming that these genes are not influenced by parasitic infection in septic mosquito guts or carcasses and suggesting the occurrence of a bacteria-independent induction by parasite infection.

Interestingly, the transcript abundance of these genes was not modulated by infection of either septic or aseptic mosquitoes with the rodent malaria parasite *P. berghei*, suggesting a possible functional role in parasite species-specific immune defenses (Figure 2.3 C, D). *P. falciparum* and *P. berghei* exhibit stark differences in their development within the mosquito that may explain the *P. falciparum*-specific induction observed in this study (Tahar et al. 2002). The lack of transcript abundance modulation in parasite-infected septic mosquitoes is interesting and may indicate that these genes are also regulated by bacteria in a way that counteracts or masks induction by *P. falciparum* when compared to the non-infected mosquitoes. Interestingly, CLIPC2 was previously reported to be induced in the midguts of mosquitoes fed heat-killed *E. coli,* suggesting that CLIPC2 is upregulated in both a bacteria-dependent and bacteria-independent but parasite-

dependent manner (Kumar et al. 2010). The differential regulation of CLIPC2 by *P. berghei* and *P. falciparum* infection of aseptic mosquitoes does support parasite-mediated regulation rather than an exclusive dependence on bacteria (Figure 2.3 D). Other CLIP C family members, such as the *Drosophila* proteins PERSEPHONE and GRASS, positively regulate two distinct TOLL pathway serine protease cascades (Ashok 2009). Thus, it is possible that CLIPC2 is involved in an innate immune cascade in *A. gambiae*. Phylogenetically, SRPN7 lies within a mosquito-specific expansion cluster and does not cluster with the serpins that have been shown to regulate melanization, and its potential role in innate immunity therefore remains elusive (Michel et al. 2005). Overall, our data suggest that CLIPC2 and SRPN7 are being induced by *P. falciparum* infection through a bacteria-independent mechanism.

Table 2.2 Microarray Data Analyzed in this Study

Gene Name	AGAP-ID	Aseptic Mg	Aseptic Car	Septic Mg	Septic Car
SOD-Cu-Zn	AGAP001623	0.4242	-0.2568	0.9679	-0.7625
SCRASP1	AGAP005625	-0.4064	-0.3171	0.8802	-0.1738
SPZ2	AGAP006483	0.4037	0.4167	0.7525	0.5334
TEP1	AGAP010815	0.2604	-0.1436	0.7241	
LRRD1	AGAP000360	1.1453	0.8669		-1.3309
LRRD18	AGAP000054	1.3001			
FBN34	AGAP001554	1.0366	0.9767	-0.5955	
FBN	AGAP010772	1.8590			
SCRB5	AGAP002738	3.1743			
DSCAM	AGAP007092	1.4899	1.0346	-0.9161	
CLIPC2	AGAP004317	0.9644	0.7252	0.5038	-0.9215
SRPN7	AGAP007693	4.1573			

Table S2. Microarray data analyzed in this study. (For full version, see supplement in Blumberg *et al*. 2013). Microarray assayed Log2 transformed transcript abundance ratio of corresponding genes discussed in section 2.5 using a cutoff of 0.75 for upregulation and -0.75 for downregulation. The "AGAP-ID" column lists the Vectorbase identifier for a particular gene (http://www.vectorbase.org). The following four columns display Log2 ratio values from either the midgut or carcass between the aseptic and septic treatments. Mg = midgut, Car = carcass.

Figure 2.3 Tissue-specific expression of *SRPN7* and *CLIPC2* after *Plasmodium* infection.

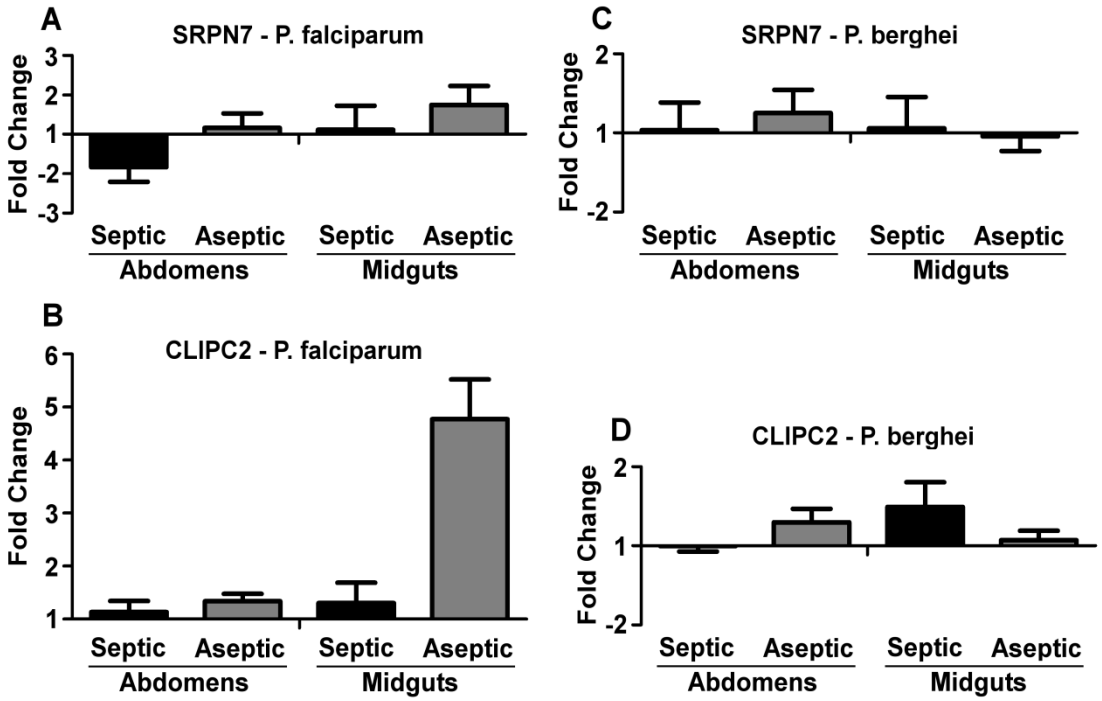

Figure 2.3: Tissue-specific expression of SRPN7 and CLIPC2 after Plasmodium infection. Fold change in transcript abundance of (A) *SRPN7* and (B) *CLIPC2* at 24 h post-*P. falciparum* infection. (C) Fold change in expression of *SRPN7* and (D) *CLIPC2* at 24 h post-*P. berghei* infection. Bars represent the mean-fold change in transcript abundance of *SRPN7* and *CLIPC2* between tissues (Midgut/Abdomen) and treatments (Septic/Aseptic) when compared to naïve blood-fed controls of the same tissue/treatment. Data are from three independent biological replicates, and error bars represent the standard error of the mean. Statistical analysis performed by Mann-Whitney test comparing the dCT values of infected to uninfected samples of the same tissue/treatment type resulted in no significant difference between any of the comparisons. There was also no significant difference between tissues when comparing transcript abundance of aseptic to septic samples of the same tissue compartment. These data were processed according to Livak and Schmittgen 2001 (Livak and Schmittgen 2001).

2.7 SRPN7 influences mosquito susceptibility to Plasmodium infection

We have shown that SRPN7 and CLIPC2 transcript abundance changes upon *P. falciparum* infection in a bacteria-independent fashion. To investigate whether SRPN7 or CLIPC2 could modulate mosquito permissiveness to *Plasmodium* infection, we used RNAi (RNA interference)-mediated gene silencing in conjunction with *Plasmodium* infection assays under aseptic conditions. Depletion of SRPN7 resulted in a significant decrease ($P< 0.001$) in the intensity of *P. falciparum* infection in comparison to *GFP* dsRNA-injected controls (Figure 2.4 A). However, there was no significant difference in the prevalence of *P. falciparum* infection between SRPN7-depleted mosquitoes when compared to the *GFS* dsRNA-injected controls (Table 2.3).

Dipteran serpins function in diverse processes ranging from inhibition of signaling cascades, such as the TOLL pathway and the prophenoloxidase activation system, to developmental processes such as morphogenesis (Ashok 2009; An et al. 2011; Reichhart, Gubb, and Leclerc 2011). An earlier study has shown that the *Anopheles* SRPN6 has an anti-*Plasmodium* function that is dependent on the mosquito strain/species (Abraham et al. 2005). The significant reduction in *P. falciparum* infection intensity that we observed upon SRPN7 depletion suggests that this serpin functions as an inhibitor of

an anti-*Plasmodium* defense that involves a serine protease cascade. The fact that, phylogenetically, SRPN7 does not cluster with the serpins known to be involved in melanization cascades, and the knowledge that the Keele strain mosquitoes used in our study have a weak melanization response and do not melanize *P. falciparum* together suggests that SRPN7 may be regulating a previously undescribed anti-*Plasmodium* mechanism. Alternatively, the role of SRPN7 in the Keele strain melanization response could be involved in parasite clearance as opposed to direct melanization (Abraham et al. 2005).

Although CLIPC2 was upregulated nearly 5-fold in response to *P. falciparum* infection in aseptic midguts, RNAi-mediated depletion of its transcript resulted in no statistical difference in the intensity of *P. falciparum* infection, although there was a slight increase in the overall infection intensity (Table 2.3). This result may suggest a predominant role for CLIPC2 in some non-defense-related process that occurs during *Plasmodium* infection, such as tissue repair or the stress response. Alternatively, an anti-*Plasmodium* defense mediated by CLIPC2 might regulate a single component within a plethora of defenses normally elicited by the endogenous microflora, which we have previously shown can have a significant effect on the intensity of *Plasmodium* infection (Dong, Manfredini, and Dimopoulos 2009).

We and others have previously shown that different mosquito immune responses are involved in the defense against infection with the two malaria

parasite species *P. falciparum* and *P. berghei*. The IMD pathway has been associated with defense against *P. falciparum,* whereas the TOLL pathway is associated with defense against *P. berghei* (Cirimotich et al. 2010). We have also shown that SRPN7 and CLIPC2 transcripts are induced in aseptic mosquito midguts upon infection with *P. falciparum* but not *P. berghei*. To investigate whether SRPN7 and CLIPC2 are regulating a general anti-*Plasmodium* defense or alternatively *Plasmodium*-species-specific defenses, we performed RNAi-mediated gene silencing upon infection with *P. berghei*. Interestingly, independent depletion of either SRPN7 or CLIPC2 resulted in no statistical difference in the intensity of *P. berghei* infection when compared to control *GFP* dsRNA-injected control mosquitoes (Figure 2.4 B, Table 23). This result supports the disparity between the mosquito immune response to either *P. falciparum* or *P. berghei* infection and underscores the importance of utilizing the human malaria parasites in mosquito infection studies in order for the results to be of relevance to human disease transmission.

Figure 2.4 *Plasmodium* infection intensity in aseptic mosquitoes after depleting *SRPN7* or *CLIPC2* through RNAi gene silencing.

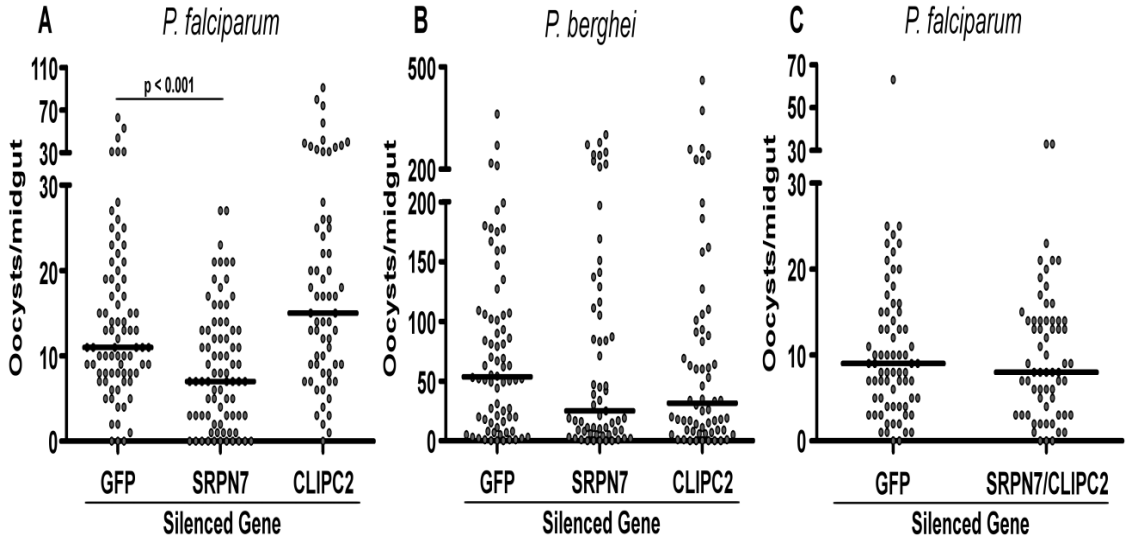

Figure 2.4: Plasmodium infection intensity in aseptic mosquitoes after depleting SRPN7 or CLIPC2 through RNAi gene silencing. (A) *P. falciparum* infection intensity following RNAi-mediated depletion of *SRPN7* (Dunn's post test, $p < 0.05$) and *CLIPC2* (Dunn's post test, $p > 0.05$). (B) *P. falciparum* infection intensity following double RNAi-mediated depletion of *SRPN7* and *CLIPC2* ($p = 0.87$). (C) *P. berghei* infection intensity following RNAi-mediated depletion of *SRPN7* and *CLIPC2* (Kruskal-Wallis test $p = 0.42$). Circles represent the number of oocysts from a single midgut; horizontal black bars represent the median oocysts in each RNAi treatment. Three independent biological replicates were pooled, and significance was determined by a Kruskal-Wallis test followed by Dunn's post-test in the case of multiple comparisons. Statistical analysis of the double RNAi knockdown was performed using a Mann-Whitney test. RNAi treatments were compared to *dsGFP*-injected control mosquitoes.

Table 2.3 Summary Statistics from *Plasmodium* Infection Assays in Figure 2.4

A. Summary Statistics from Figure 2.4 (A)

P. falciparum	GFP	SRPN7	CLIPC2
N	79	79	63
Mean	14.39	8.56	20.38
Median	11	7	15
Prevalence	94.5	83.5	97.5
% Change (Median)		-36%	+36%

B. Summary Statistics from Figure 2.4 (B)

P. berghei	GFP	SRPN7	CLIPC2
N	74	61	62
Mean	74.88	72.74	72.23
Median	53.5	25	31.5
Prevalence	96%	92%	90%
% Change (Median)		-53%	-41%

C. Summary Statistics from Figure 2.4 (C)

P. falciparum	GFP	SRPN7/CLIPC2
N	71	63
Mean	10.55	10.02
Median	9	8
Prevalence	97%	95%
% Change (Median)		-11%

Table 2.3: Summary statistics data from Figure 2.4. Supplementary data from *Plasmodium* infection assays displayed in Figure 4. Includes number of samples assayed, mean, median, % change, and prevalence of oocysts.

2.8 SRPN7 and CLIPC2 may function in the same SP cascade

Since serpins and Clip-domain serine proteases function together as signal transducers and inhibitors in proteolytic signaling cascades, we performed a double knockdown assay of SRPN7 and CLIPC2 in aseptic *P. falciparum*-infected mosquitoes to provide a baseline indication as to whether these factors could be functioning in the same cascade, and thereby reciprocally influence their knockdown infection phenotypes. Interestingly, co-silencing of the two genes abolished the effects on *P. falciparum* infection that was observed when each gene was silenced independently (Figure 2.4 C, Table 2.3). Although the potential for a direct interaction between a serpin and serine protease should be examined by a rigorous biochemical analysis, this experiment, taken together with the bacteria-independent opposite effects of SRPN7 and CLIPC2 depletion on susceptibility to *P. falciparum* infection, suggest that SRPN7 and CLIPC2 maybe operating in the same cascade that regulates anti-*Plasmodium* defense. Alternatively, SRPN7 and CLIPC2 could be negative and positive regulators, respectively, of separate processes and thus the result could merely be explained by a canceling effect of silencing both transcripts. Without a biochemical analysis addressing interaction between the two proteins, it may be more accurate to assume that

these genes are negative and positive regulators, possibly of the same cascade or independent cascades.

2.9 CLIPC2 and SRPN7 influence systemic bacterial infection and the midgut microbiota

We have previously shown that anti-*Plasmodium* factors also play versatile functions in antibacterial defense and wanted to investigate whether SRPN7 or CLIPC2 could play a role in the mosquito's ability to fight systemic bacterial infection, or in the control of its midgut microbiota. While RNAi-mediated depletion of SRPN7 or CLIPC2 did not affect the mosquito's survival upon experimental infection with *S. aureus*, mosquitoes depleted of CLIPC2 showed increased survival when infected with *E. coli*, suggesting that CLIPC2 may be a host factor for this bacterium (Figure 2.5 A, B). The mosquito's midgut microbiota needs to be under continuous immune control to avoid an over-proliferation that could be detrimental to the insect. We have previously shown that factors of the IMD immune pathway play a crucial role in controlling the midgut microbiota, and conversely, that the microbiota is responsible for priming basal immune activity (Clayton et al. 2013; Dong, Manfredini, and Dimopoulos 2009). Surprisingly, independent silencing of SRPN7 andCLIPC2 resulted in a significant decrease of the mosquito's

midgut microbiota, suggesting that these putative immune factors act as agonists of the mosquito's natural midgut bacteria, through an unknown mechanism (Figure 2.5 C). The midgut microbiota are predominately Gram-negative, and the decrease in the midgut bacteria in response to the silencing of CLIPC2 corroborates the increase in survival that was observed in *E. coli*-challenged mosquitoes depleted of CLIPC2 (Jenny M Lindh, Terenius, and Faye 2005). Whereas silencing of SRPN7 resulted in a decrease in the mosquito's midgut microbiota, silencing of this gene had no effect on survival after systemic bacterial challenge, suggesting that the function of SRPN7 may be associated with the midgut as opposed to the fat body. Although we have hypothesized that SRPN7 and CLIPC2 operate in the same or different serine protease cascades to activate defense mechanisms, the effects of SRPN7 and CLIPC2 depletion on resistance to bacterial infection and the microbiota, respectively, cannot be fully explained by this model and suggests that these two factors may be associated with multiple and functionally diverse serine protease cascades. Nevertheless, our results further corroborate the functional unrelatedness of SRPN7 and CLIPC2 with the bacteria-dependent IMD pathway-mediated anti-*Plasmodium* defense system and highlight the functional versatility and complexity of mosquito immune factors.

Figure 2.5 Influence of *SRPN7* and *CLIPC2* silencing on mosquito resistance to bacterial challenge and midgut microbiota proliferation.

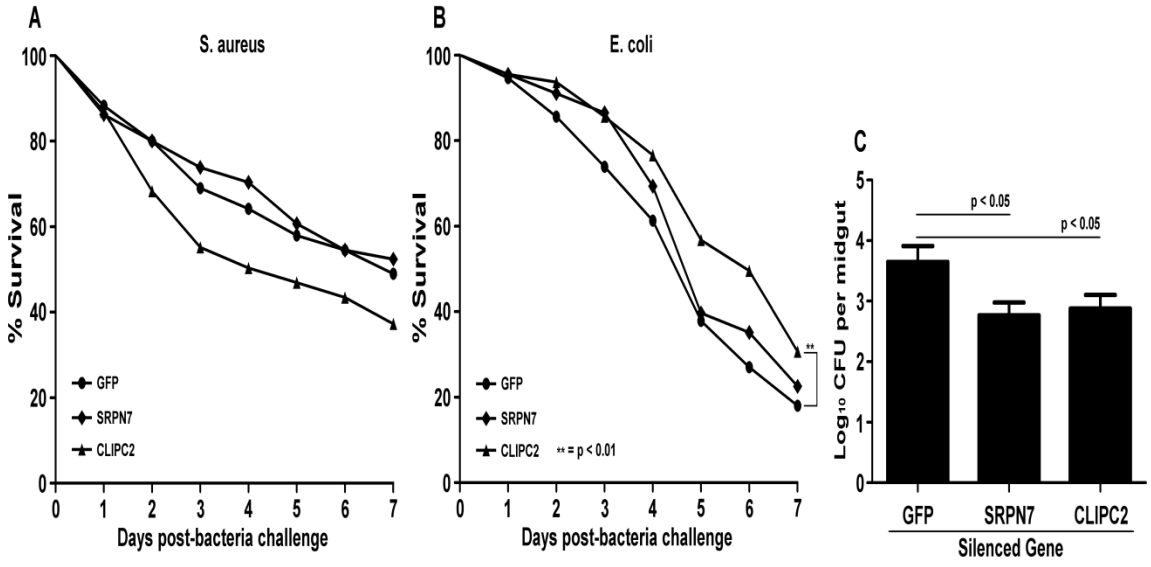

Figure 2.5: Influence of SRPN7 and CLIPC2 silencing on mosquito resistance to bacterial challenge and midgut microbiota proliferation. Adult female mosquitoes were subjected to RNAi-mediated depletion of *SRPN7* or *CLIPC2* transcripts and then challenged with (A) either Gram-positive *Staphylococcus aureus* or (B) Gram-negative *Escherichia coli* bacteria. Depletion of *SRPN7* ($p = 0.56$) or *CLIPC2* ($p = 0.028$) had no effect on the survival of mosquitoes challenged with (A) *S. aureus*, whereas there was a significant increase($p < 0.01$) in the survival of *CLIPC2*-depleted mosquitoes challenged with (B) *E. coli* but not *SRPN7*-depleted mosquitoes ($p = 0.18$). For both (A) and (B), data were pooled from three independent biological replicates (for A, n=145; for B, n=111), and a control group injected with *dsGFP* RNA was included in each replicate. Statistical significance was determined using Kaplan-Meier survival analysis with a log-rank test using Bonferonni's correction for multiple comparisons (significance = $p < 0.025$.(C) RNAi-mediated gene silencing of *SRPN7* or *CLIP2* resulted in a significant decrease ($p < 0.05$) in the colony forming units (CFU) of cultivable midgut bacteria when compared to *dsGFP*-injected control mosquito midguts. Data were pooled from three independent biological replicates (n=27 for each dsRNA group), and statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison test. Error bars represent the standard error of the mean.

2.10 SRPN7 and CLIPC2 do not regulate the IMD pathway

Although serpins and clip serine proteases have been identified as regulators of the TOLL pathway, it is unclear whether similar cascades are involved in regulating the activation of the IMD pathway. The TOLL pathway is primarily responsible for immunity against the rodent parasite *P. berghei,* whereas the IMD pathway is associated with immunity against the human parasite *P. falciparum* (Cirimotich et al. 2010). Since SRPN7, and to some degree CLIPC2, appear to modulate *P. falciparum* infection intensity, we hypothesized that these genes could be involved in regulating the IMD pathway. In order to determine whether SRPN7 or CLIPC2 is involved in activating the IMD pathway, we used qRT-PCR to measure the abundance of three IMD-pathway-regulated, anti-*Plasmodium* gene transcripts (TEP1, FBN9, LRRD7) at 24, 48, 72, and 96 hours following RNAi-mediated gene silencing of SRPN7 or CLIPC2 in aseptic mosquitoes (Table 2.1). We chose to monitor the expression of these genes over 4 days because of the previously reported temporal regulation of IMD pathway-driven gene transcript abundance (Frolet et al. 2006). The silencing efficiency over 4 days averaged 62% for SRPN7 and 59% for CLIPC2, respectively (Figure 2.6 A, B). Depletion of either SRPN7 or CLIPC2 did not influence the transcript abundance of TEP1, FBN9, or LRRD7 over the course of the experiment

when compared to GFP dsRNA-injected control mosquitoes (Figure 2.6 C, D). These three potent anti-*Plasmodium* genes were previously found to be upregulated by the IMD pathway upon silencing of the negative regulator CASPAR and upon overexpression of the IMD pathway transcription factor REL2 (Garver, Dong, and Dimopoulos 2009). In our earlier studies we have also shown that the abundance of SRPN7 and CLIPC2 transcripts is not regulated by the IMD pathway (Garver, Dong, and Dimopoulos 2009). FBN9 is induced by the native microbiota in the mosquito midgut, and all three genes are involved in controlling its microbiota as well as in systemic bacterial challenge (Dong, Manfredini, and Dimopoulos 2009; Dong et al. 2006). Furthermore, none of these genes were upregulated in the *P. falciparum*-infected aseptic midguts. In the absence of bacteria, SRPN7 and CLIPC2 were upregulated in the *P. falciparum*-infected aseptic midguts, and depletion of SRPN7, and to some degree CLIPC2, modulated the intensity of *P. falciparum* infection, yet these genes do not appear to regulate the expression of anti-*Plasmodium* factors through the IMD pathway. These findings suggest that bacteria- and IMD pathway-independent anti-*P. falciparum* defenses exist, and they underscore the complexity of the mosquito's anti-*Plasmodium* immune mechanisms.

Figure 2.6 *SRPN7* or *CLIPC2* depletion has no effect on the expression of IMD pathway-regulated anti-*P. falciparum* genes.

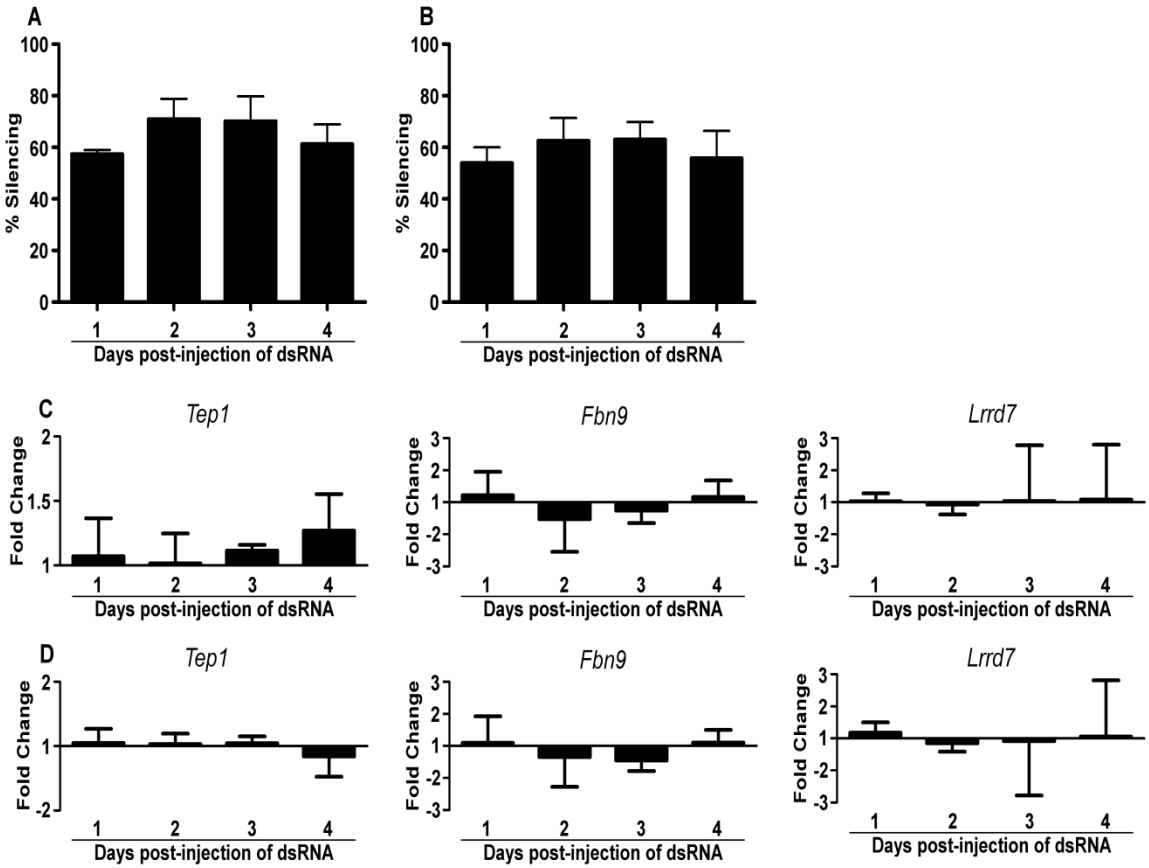

Figure 2.6: SRPN7 or CLIPC2 depletion has no effect on the expression of IMD pathway-regulated anti-P. falciparum genes. **(A)** Silencing of *SRPN7* and *CLIPC2* was measured over a period of 4 days by qRT-PCR. Fifteen midguts, from aseptic mosquitoes, were pooled on each day post-injection, and the results represent the mean silencing for two independent biological replicates. Error bars represent the standard error of the mean. Expression of *TEP1, FBN9,* and *LRRD7* genes following single knockdown of (C) *SRPN7* or (D) *CLIPC2*. Bars represent the -fold change in expression of the listed genes on days 1-4 post-dsRNA injection, as compared to *dsGFP*-injected controls. qRT-PCR was used to assess changes in expression of the genes indicated above each graph. Error bars represent the standard error of the mean for three biological replicates. Statistical analysis was performed at each time point by one-way analysis of variance (ANOVA) followed by Dunnett's post-test to account for multiple comparisons; all genes showed no significant difference in expression when compared to *dsGFP*-injected controls (not depicted).

2.11 Discussion

The *Anopheles* mosquito's anti-*Plasmodium* defense system is actively engaged in limiting parasite infection of the midgut epithelium by mounting immune responses against the ookinetes in the midgut lumen and epithelium (Dong et al. 2011). While these immune responses have been shown to be regulated to some extent by midgut microbiota-mediated activation of the IMD pathway, we show here for the first time that other, as yet uncharacterized, microbiota- and IMD pathway-independent immune responses also participate in limiting *P. falciparum* infection. The potential affiliation of SRPN7 and CLIPC2 with a serine protease activation cascade suggests that these genes are controlling the activation of an effect mechanism, rather than representing effectors themselves. The regulation and parasite killing mechanism of these defenses appear to be quite different from those previously characterized since (a) SRPN7 and CLIPC2 are not regulated by, nor do they regulate, the IMD pathway and (b) they act against *Plasmodium* independently of the midgut microbiota. The observation that SRPN7 and CLIPC2 were only regulated in the *P. falciparum*-infected aseptic midguts, strongly suggests that an upstream pattern recognition molecule is sensing *P. falciparum* and culminating in the activation of an undescribed pathway. Alternatively, a molecule upstream of SRPN7 and CLIPC2 could be

sensing damage to the midgut epithelium mediated by *P. falciparum* invasion. SRPN7 and CLIPC2 were neither induced by nor involved in anti-*P. berghei* defense, suggesting an association with defense against *P. falciparum* and demonstrating the ability of the mosquito immune system to discriminate between infections of closely related pathogens. A *P. falciparum*-specific defense pathway could be exploited in a translational approach to control *Plasmodium* in the mosquito, as opposed to the human host. A biochemical analysis of their interacting partners will be necessary to confirm that these molecules are true partners and that they regulate the same effector mechanism. In summary, we have discovered SRPN7 and CLIPC2 in the bacteria-independent, *Plasmodium* infection-responsive transcriptome and demonstrated the existence of IMD pathway-independent defenses against *P. falciparum*.

Chapter 3: Fungal-mediated enhanced *Plasmodium* infection in *Anopheles* mosquitoes

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3.1: Abstract

Anopheles mosquitoes are vectors of *Plasmodium* parasites that cause human malaria, a disease of utmost public health importance. The mosquito holobiont, which is a combination of the mosquito immune system and associated microbes, determines the outcome of *Plasmodium* infection. Mosquito-associated bacteria are capable of modulating *Plasmodium* infection by stimulating the mosquito immune system, by physically impeding parasite invasion, or by antagonizing parasites through the secretion of anti-pathogen effector molecules. While bacteria have been heavily investigated for their ability to block *Plasmodium* infection in the mosquito, little is known about the role of fungi. Here we report the isolation of the common ascomycete fungus, *Penicillium chrysogenum,* from the midgut of a field-caught *Anopheles* mosquito. *P. chrysogenum* introduced through an artificial sugar meal does not affect *A. gambiae* survival. Surprisingly, we discovered that ingested *P. chrysogenum* results in increased *Plasmodium* infection of the mosquito. *P. chrysogenum* enhances infection even in

genetically modified *A. stephensi* that are normally refractory to *P. falciparum*. *P. chrysogenum* does not suppress some of the known anti-*P. falciparum* effector mechanisms at the transcript or protein level. A heat-stable, soluble factor associated with *P. chrysogenum* may be responsible for the increase in mosquito susceptibility to *Plasmodium*. To our knowledge, this is the first report of a fungus modulating *Plasmodium* infection in the mosquito midgut, and may have epidemiological implications in relation to malaria transmission.

3.2: Rational and Hypothesis

There has been renewed interest in characterizing the *Anopheles* microbiota (bacteria) due to its strong influence on the course of *Plasmodium* infection in the mosquito. The midgut microbiota modulate anti-*Plasmodium* defense by stimulating IMD/Rel-2 mediated production of anti-*Plasmodium* effector molecules (Meister et al. 2009; Garver, Dong, and Dimopoulos 2009; Dong, Manfredini, and Dimopoulos 2009). Bacteria introduced by way of an artificial blood meal antagonize *P. falciparum* infection through physical or chemical processes (Bahia et al. 2014; Dong, Manfredini, and Dimopoulos 2009). In fact, the microbiota have been heavily investigated for their ability

to chemically impede parasites in *Anopheles*. Certain bacteria from the genera *Chromobacterium*, *Enterobacter*, and *Serratia* restrict parasite development in the midgut through secreted anti-pathogen factors and other mechanisms (Jose Luis Ramirez, Short, et al. 2014; Bahia et al. 2014; Cirimotich et al. 2011). The midgut microbiota have important public health implications as they could be exploited in translational approaches for *Plasmodiu*m and vector control. This has spurred numerous lab and field investigations to identify and characterize mosquito-associated bacteria.

Unlike the microbiota, much less is known about the internal mosquito mycobiome. Most studies have explored entomopathogenic fungi for control of vector populations (Fang et al. 2011; Bukhari, Takken, and Koenraadt 2011). We isolated an ascomycete fungus from the midgut of an *Anopoheles* mosquito during a collection trip to Puerto Rico. Visual inspection of conidial structure by light microscopy in combination with DNA sequencing of the ribosomal ITS region identified this fungus as a strain of *Penicillium chrysogenum* (Schoch et al. 2012). *P. chrysogenum* is found globally in temperate and subtropical regions, and it as well as other *Penicillium* species have been isolated from mosquitoes (da S Pereira et al. 2009; da Costa and de Oliveira 1998). *P. chrysogenum* is considered non-pathogenic, although some strains are capable of producing harmful mycotoxins (Hidalgo et al. 2014). We postulated that mosquitoes could be acquiring *P. chrysogenum* through

feeding on contaminated sugar or water, and investigated the implications in the context of *Plasmodium* infection of the mosquito midgut. The TOLL and JAK-STAT pathways participate in mosquito anti-fungal defense, and we reasoned that *P. chrysogenum* could modulate anti-pathogen defense mechanisms in the mosquito.

3.3: Materials and Methods

3.3.1 Mosquito Rearing, RNA Isolation, and cDNA Synthesis

A. gambiae Keele strain, wild type *A. stephensi* Liston strain, and transgenic *A. stephensi* (Cp-REL2) mosquitoes were maintained on a 10% sucrose solution with 12-h light/dark cycles at 27^{o}C and 80% humidity (Hurd et al. 2005; Crampton, Beard, and Louis 1997; Dong et al. 2011). At specific time points, mosquitoes were anesthetized on ice, and either whole mosquitoes (heads removed) or specific tissues were dissected and collected. RNA was extracted from tissues using TRIzol® (Life Technologies™). Following treatment with DNase (Ambion), RNA yields were quantified using the Nanodrop 2000 (ThermoFisher) and cDNA was synthesized from

extracted RNA using the Oligo-DT primer and M-MLV Reverse Transcriptase (Promega).

3.3.2 *Genomic DNA Extraction, PCR, and Sequencing*

P. chrysogenum conidia were collected as described in *3.3.4*, resuspended in 150uL of sterile 1x PBS, homogenized (Bullet Blender, Next Advance) with 0.5-mm glass beads for 2 minutes, and the homogenate was incubated at 70^{o} C for 15 minutes to inactivate residual enzymes and prevent sample degradation . A Phenol:Chloroform (Sigma) extraction of nucleic acids was performed on this homogenate, and following RNase (Qiagen) treatment, DNA yields were quantified using the Nanodrop 2000 (ThermoFisher). Universal fungal primers were used to amplify a region of ribosomal DNA, PCR products were separated by electrophoresis in 1% agarose gel, the target amplicon was column purified (Qiagen), sent to the JHMI genomics core for Sanger sequencing, and the returned nucleotide sequence was searched against a non-human nucleotide database using BlastN™ (Schoch et al. 2012). See Table T1 for primers.

3.3.3 qRT-PCR

The Primer 3 Program (http://frodo.wi.mit.edu) was used to design the qRT-PCR primer REL2 whereas other primers are previously described (Blumberg et al. 2013; Jose Luis Ramirez, Garver, et al. 2014). Real-time quantitative PCR (qRT-PCR) was used to assess transcript abundance, which was quantified with Sybr Green PCR Master Mix (Applied Biosystems) using the ABI StepOnePlus Real-Time PCR System and ABI StepOne Software. PCR reactions were performed at least in duplicates, melting curve analysis was used to analyze primer specificity, and the same cycle threshold value was applied across all experiments. Transcript abundance of target genes were first normalized to the within sample transcript abundance of the mosquito ribosomal *S7* gene, and fold changes between samples were determined using the ΔΔct method. See Table T1 for primers.

3.3.4 Culturing of P. chrysogenum and Collection of Conidia

P. chrysogenum was initially isolated on Yeast Peptone Dextrose Agar (YPDA) (Sigma), and subsequent cultures were maintained on Sabouraud Glucose Agar 4% (SGA)(SIGMA). Briefly, 4mL of Sabouraud Dextrose Broth (SDB), was inoculated with *P. chrysogenum* which was shaken at 27-30^{o} C for up to 72 hours. These *P. chrysogenum* cultures subsequently were kept at 4^{o}

C and used to inoculate SGA plates for up to 3 weeks. Using Sterile technique, 100uL of broth was added to SGA, and *P. chrysogenum* was cultured at 27° C for 1-2 weeks or until the surface became confluent with conidia Conidia were collected by flushing plates with 1x PBS containing 0.1% Tween 80 (Sigma) and by gently scraping the conidia into solution. Glass wool filtration of this solution removed residual agar and mycelium, and the filtered conidia were centrifuged at 2,000 RPM for 10 minutes. The supernatant was discarded and the conidia pellet was washed with 50mL of sterile 1x PBS. After repeating centrifugation, the pellet was resuspended in 1mL of sterile 1x PBS, serially diluted, and a hemocytometer (Neubaur) was used to enumerate conidia by light microscopy (40x). Concentrated conidia were added to 25% glycerol for long-term storage at -80°C.

3.3.5 Preparation of P. chrysogenum Filtrate

Two confluent 175mm plates (BD) of conidia were used for each preparation of filtrate. After washing and centrifuging, the PBS supernatant was discarded and the conidia pellet was resuspended in 1mL of sterile 10% sucrose solution. This suspension was vortexed briefly for 20 seconds and then conidia were pelleted by centrifugation at 2,000 RPM for 10 minutes. After centrifugation, the supernatant was collected in a 1mL syringe and

passed through a 0.2 micron filter (Millipore). For heat inactivation, filtrates were incubated at 95° C for 2 hours. A small aliquot of filtrate was cultured on SGA plates to confirm the absence of live fungi.

3.3.6 *Conidia or Filtrate Introduced to Anopheles by Sugar Meal*

3-4 day old adult female *Anopheles* mosquitoes were briefly starved by removing 10% sucrose for 4-8 hours. Conidia were added to sterile 10% sucrose solution to obtain a final volume of 4mL, the solution was absorbed to cotton and placed in a location accessible to mosquitoes. For filtrate, approximately 1mL of either active or heat-inactivated filtrate was absorbed to cotton. Mosquitoes were allowed to feed on conidia or filtrate for 48 hours over the span of 2 nights. Unless indicated otherwise, conidia were administered once whereas fresh filtrate solutions were administered at days 1 and 2. 48 hours after administering conidia or filtrate, the cotton pads were removed and replaced with sterile 10% sucrose.

3.3.7 Survival of A. gambiae Fed P. chrysogenum Conidia

3-4 day old adult female *A. gambiae* were allowed to feed on *P. chrysogenum* conidia as described in section *3.3.6*. At day 0, mosquitoes were given sterile 10% sucrose solution that was changed every 2 days. Survival of mosquitoes was monitored for 14 days and dead mosquitoes were removed as necessary.

3.3.8 Quantification of Endogenous Mosquito Microbiota and P. chrysogenum in the *Anopheles* Midgut

Colony forming units (CFU) from mosquito midguts were quantified in control untreated, fungi-fed, and filtrate-fed mosquitoes as described (Dong, Taylor, and Dimopoulos 2006; Dong, Manfredini, and Dimopoulos 2009). 3-4 day old female *A. gambiae* were fed fungi or filrates and at 48 hours post-feeding, female mosquitoes were collected, surface-sterilized in ethanol, and washed with 1x PBS, and their midguts were dissected in sterilized 1x PBS. Collected midguts were homogenized, and serial dilutions of homogenate were added to LB agar plates. After incubation for 2-3 days at 27^{o}C under aerobic conditions, the CFUs per plate were counted, and a titer of CFU/midgut was calculated(Dong, Taylor, and Dimopoulos 2006; Dong, Manfredini, and Dimopoulos 2009). *P. chrysogenum* colony forming units (CFU) from midgut samples were quantified similarly except midgut samples

were cultured on PGA plates containing 75 µg/mL gentamicin sulfate (Quality Biological) and 100 units (µg)/mL of penicillin-streptomycin (Invitrogen). Plates incubation for up to 4 days at 27°C and were inspected every day for hyphal nuclei that were enumerated to determine the fungal CFU/midgut.

3.3.9 Plasmodium Challenge

P. falciparum and *P. berghei* challenges were accomplished following a standard protocol (Dong et al. 2006). For *P. falciparum* infection: 48 hours post-feeding on fungi or filtrate, mosquitoes were fed on NF54W strain gametocytes in human blood through a membrane feeder at 37°C. Unfed mosquitoes were removed within the first day post-infection, and engorged mosquitoes were maintained at 27°C for up to 8 days. For *P. berghei* infection: Two days post-feeding on fungi or filtrate, mosquitoes were allowed to feed on Swiss Webster mice infected with the WT Anka 2.34 strain of the parasite. Unfed mosquitoes were removed within the first day post-infection, and engorged mosquitoes were maintained at 19°C for 14 days. *P. falciparum*- and *P. berghei*-infected mosquito midguts were dissected and stained with 0.1% mercurochrome, and oocyst numbers were counted using a light microscope (Olympus).

3.3.10 Antibiotic Treatment for P. falciparum Challenge

For antibiotic treatment, adult female mosquitoes were collected post-eclosion and given a sterile 10% sucrose solution containing 75 µg/mL gentamicin sulfate (Quality Biological) and 100 units (µg)/mL of penicillin-streptomycin (Invitrogen). Treatment was carried out for 3 days, and antibiotic-containing sucrose was changed daily to ensure adequate elimination of bacteria. To validate the efficiency of antibiotic treatment, midguts from control untreated and antibiotic treated mosquitoes were subjected to CFU assays (Blumberg et al. 2013). Where indicated, conidia or filtrate solutions were supplemented with antibiotics. Antibiotic cohorts were maintained on antibiotic-treated sucrose following a blood meal.

3.3.11 Ex Vivo P. falciparum Ookinete Counts

For human malaria parasite infection assays, mosquitoes were provided blood containing gametocytes of *P. falciparum* NF54W as described in *3.3.9*. Ookinete determination was carried out by dissecting midguts 24-28 hours post-bloodmeal. Individual midguts were gently homogenized in PBS by pipetting in 40 ul of PBS in one well of 96-well plate, using low-retention hydrophobic tips. Homogenates (10 ul) were spotted on a slide, air-dried and fixed in methanol,

followed by Giemsa staining. Ookinetes were enumerated by light microscopy using an oil (100x) lens (Olympus).

3.3.12 In Vitro Ookinete Inhibiton Assays

Female Swiss Webster mice (6-8 weeks old) were infected with genetically modified *P. berghei* that express Renilla Luciferase under the control of an ookinete-specific promoter. Starting from 3 days post infection, smears were made and exflagellation assays performed. When at least 20 exflagellation events were recorded in a 20x field, mice were bled by heart puncture using a heparinized needle, blood diluted in 10 volumes ookinete media (RPMI 1640, 10% FBS, 50 mg/ml hypoxanthine, 2 mg/ml NaHCO3, pH 8.3) with 4% mouse RBC lysate in 24 well plates each well containing a final volume of 500 ml. For 96 well plate (Costar 3595, Corning NY USA) cultures, the final volume in each well was 100 ml. To assay the ookinete inhibitory effect of *P. chrysogenum*, conidia or filtrate were added to each well in triplicate along with experiment-matched controls. To control for background expression of renilla luciferase, cultures were maintained in ookinete media adjusted to pH 7.0 (ookinete development requires a pH of 8.3). Ookinete cultures were incubated at 19°C for 26-28 hr and culture material in each well transferred into an eppendorf tube. The culture

material was spun at 1,800 rpm for 4 min and the supernatant was discarded. The pellet was processed immediately or stored at -80°C pending further analysis.

3.3.13 Luciferase Assay

The luciferase assay was performed using the renilla luciferase assay system (E2810, Promega USA) according to the manufacturer's instructions. 100 ml 1X lysis buffer was added to the pellet from above and gently vortexed and incubated at RT for 15 min. The lysate was spun at 14,000 rpm for 2 min and supernatant transferred to a clean epperndorf tube and placed on ice. 20 ml of supernatant was added to 100 ml of luciferase assay buffer, mixed by pipetting and the sample read immediately on the 20/^{20n} Luminometer (Turner Biosystems, USA) at 10 sec integration. Percentage inhibition of ookinete development was calculated by subtracting the blanks and expressing the luciferase units as a percentage of controls (no bacteria or drug).

3.3.14 In vitro Nitration Assay

Twenty-four hours after infection, mosquito midguts were dissected and five midguts per group were collected. Midguts were fixed in 4% paraformaldehyde/1% glutaraldehyde and washed with PBS. Then, were homogenized using a pestle in a PBS buffer containing 1 mM H_2O_2, 1 mM $NaNO_2$,and100 µg of BSA. A commercial horseradish peroxidase (Invitrogen) at a final concentration of 0.3 unit/mL was used as positive control and for the negative control samples were preincubated with 5 mg/mL ofsodium azide (NaN_3). Both, samples and controls were incubated for 30 min at 37 °C. Midgut pellet was discarded and the protein concentration of the supernatant was quantified using BCA™ Protein Assay Kit (Thermo Scientific). Three micrograms of protein per sample were separated on two4-20% Tris-glycine gels. One was stained with CoomassieBlue, the other was transferred to a PVDF membrane. The membrane was incubated with 1 mMlevamisole solution for 30 min, blocked with 5% non-fat milk in TBS-T, and incubated overnight at 4°C with an anti-nitrotyrosine mouse primary antibody (clone39B6) (1:1,500) (Sigma-Aldrich). Membrane was washed with TBS-T, incubated with ananti-mouse alkaline phosphatase-linked antibody (1:5,000) (Cell Signaling) for 2 h at RT, washed with TBS-T, and developed using Western Blue ® substrate for alkaline phosphatase (Promega).

3.3.15 TEP1 Western Blot Analysis

Reduced samples were prepared in 5X Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, with 2% SDS, 20% glycerol, 5% ß-mercaptoethanol, and 0.01% bromophenol blue), heated at 70°C for 5 min, and separated on a 4-20% gradient gel. Proteins were transferred to nitrocellulose membranes and incubated in blocking buffer (1X PBS with 0.1% Tween-20, 5% BSA) for 1 hr. Blots were incubated for 2 hr in primary antibodies diluted in PBS-T, washed five times with PBS-T, then incubated in secondary antibody diluted in PBS-T for 1 hr. Membranes were washed five times with PBS-T, and blots were developed using ECL Prime Western blotting detection reagent (GE Healthcare). All incubations were performed at room temperature. Rabbit polyclonal anti-TEP1 antibody (Washington Biotechnology, Inc) was produced using recombinant TEP1 protein as previously described (Levashina et al. 2001). Following affinity purification, the antibody was verified by western blot analysis of mosquito hemolymph (1:3000) collected as previously described, although fetal bovine serum was not added to the collection solution (Rodrigues et al. 2010; Sadd and Schmid-Hempel 2006; Castillo, Robertson, and Strand 2006). Anti-rabbit (1:100,000) secondary antibodies conjugated to peroxidase were obtained from Jackson Laboratories. Densitometric analysis was performed using Image J software (NIH).

3.3.16 Confocal Fluorescence Microscopy

A. gambiae mosquito immune competent Sua5B cells were split in Schneider's medium in 6-well plates with sterile glass cover slips (22mm x 22mm) on the bottom, and allow them to grow to 80% confluency before the treatment of fungal filtration, mycophenolic acid (MPA) as positive control and PBS as negative control. Fungal filtration were prepared as above described, and part of the filtration were thereafter concentrated 10 time which is called "Fungi-filtration concentrated", and the original ones were "low concentration". About 300 ul of fungal filtration or MPA or PBS were added to each well (with additional final 1 ml of Schneider's medium), and the final concentration of MPA is 20 uM. Confocal microscopy was done according to previously reported procedures [Dong et al., 2006]. In brief, after overnight treatment with fungal filtration or MPA or PBS, the medium were removed and the Sua5B cells were washed with PBS for three times followed by fixing in 4% paraformaldehyde for one hour at room temperature. These Sua5B cells were then washed with PBS for 3 times followed by blocking with 10% goat serum in PBS for 2 h. Thereafter, Sua5B cells were incubated with Alexa Fluor 488 phalloidin (Invitrogen A12379) diluted 1:400 in 1% bovine serum albumin/PBS overnight at 4 ^{o}C. After 3 PBS washes, the cover slips with Sua5B cells removed from the 6-well plates and mounted in Prolong

Gold Antifade reagent with DAPI (Invitrogen P36931) with glass slides. Cover slips were sealed with nail polish and subjected to a Zeiss 510 system-based confocal microscopy. Ten sequential optical sections of 1 μm each were collected and only one optical section was shown.

3.3.17 Statistical Analysis

The Graphpad Prism 5 (Graphpad Prism®) software package was used to perform statistical analyses. The particular test used is indicated in the captions of each respective figure.

3.4. P. chrysogenum is non-pathogenic to A. gambiae

We isolated a strain of *P. chrysogenum* from the midgut of an *Anopheles* mosquito collected in Puerto Rico. *P. chrysogenum* has been previously isolated from mosquitoes, and just over 50% of fungi infecting field-caught *Anopheles* were identified as *Penicillium* (da Costa and de Oliveira 1998). These results suggest *P. chrysogenum* may be an opportunistic mosquito pathogen. To examine the pathogenicity of *P. chrysogenum*, we sugar-fed adult female *A. gambiae* on sucrose spiked with $1x10^9$ conidia ($2.5x10^5$/ul) and monitored mortality over a 14 day period.

Mosquitoes fed on *P. chrysogenum* showed no difference in survival compared to sugar-fed controls (Figure 3.1). We even observed fungal masses in the crop of some fungi-fed mosquitoes (Figure S1).This result demonstrates that our *P. chrysogenum* strain is not significantly pathogenic to *A. gambiae*. This finding corroborates a study reporting low overall mosquito mortality when exposed to conidia at a concentration 4x higher than we used (Maketon, Amnuaykanjanasin, and Kaysorngup 2014). Therefore, we used $1x10^9$ conidia ($2.5x10^5$/ul) as our standard dose for the following live conidia experiments.

3.5 P. chrysogenum enhances mosquito susceptibility to Plasmodium infection

To investigate whether *P. chrysogenum* could modulate mosquito permissiveness to *Plasmodium* infection, we sugar-fed *A. gambiae* on conidia for 48 hours prior to infecting them with *Plasmodium* through feeding on a blood meal containing gametocytes. Feeding on *P. chrysogenum* resulted in a significant increase in both the intensity ($P<0.0001$) and prevalence ($P<0.05$) of *P. falciparum* infection when compared to naive sugar-fed controls (Figure 3.2 A, Table 2). Fungi have been characterized for their ability to produce antibiotics, including *P. chrysogenum,*, which are used in commercial production of the antibiotic Penicillin (Wong et al. 2014). We have shown that

depletion of the midgut microbiota enhances *Plasmodium* infection in *Anopheles*, thus we hypothesized the observed increase in *P. falciparum* susceptibility could be due to a *P. chrysogenum*-mediated reduction in midgut bacteria (Dong, Manfredini, and Dimopoulos 2009). To investigate this hypothesis, we depleted *A. gambiae* midgut microbiota with a broad-spectrum antibiotic cocktail through their sugar meal, prior to infection with *P. falciparum* gametocytes (Blumberg et al. 2013).

Figure 3.1 Survival of A. gambiae fed P. chrysogenum in a sugar meal

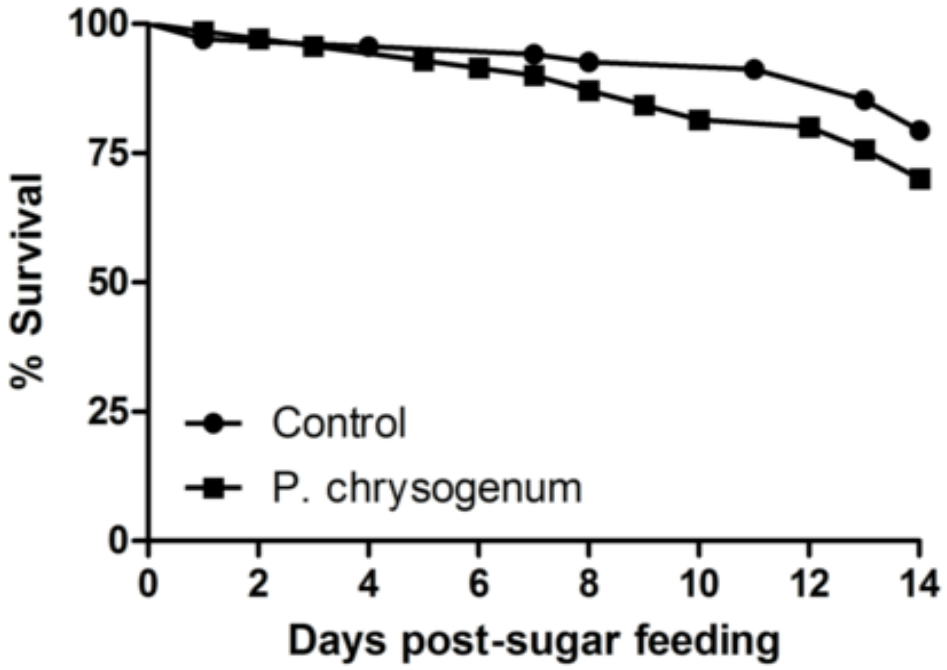

Figure 3.1: Survival of A. gambiae fed P. chrysogenum in a sugar meal. Adult females feeding on *P. chrysogenum* for 48 hours survived (70%) no differently than sugar-fed controls (79%) over 14 days of monitoring. For both, data was pooled from 4 biological replicates (N=79) and statistical significance (p = 0.18) was determined by Kaplan-Meier analysis.

Feeding on *P. chrysogenum* resulted in a significant increase in the intensity (P<0.0001) of *P. falciparum* infection in the antibiotic cohort when compared to sugar-fed controls (Figure 3.2 B, Table 2). This result suggests a bacteria-independent mechanism underlying the enhancement of *P. falciparum* infection in *A. gambiae*.

We have previously shown stark differences in mosquito immune defense against infection with the two parasite species *P. falciparum* and *P.*

berghei (Dong et al. 2006). Whereas the IMD pathway defends against *P. falciparum*, the TOLL pathway is associated with defense against *P. berghei*. To investigate whether *P. chrysogenum* is inhibiting general anti-*Plasmodium* defense or parasite-species-specific defenses, we sugar-fed *A. gambiae* on conidia for 48 hours prior to infection with *P. berghei*. Feeding on *P. chrysogenum* resulted in a significant increase in both the intensity ($P<0.05$) and prevalence ($P<0.05$) of *P. berghei* infection (Figure 3.2 C, Table 2). This suggests *P. chrysogenum* may be inhibiting several anti-*Plasmodium* defense mechanisms. The JAK-STAT pathway participates in anti-fungal and anti-viral defense, and we previously showed that the entomopathogenic fungus *B. bassiana* is capable of priming JAK-STAT anti-viral defenses in *Aedes aegypti* (Dong, Morton, et al. 2012). The TOLL pathway also defends against fungal infection in the mosquito, but the observed increase in *Plasmodium* infection suggests *P. chrysogenum* may not prime TOLL-mediated, anti-*P. berghei* defenses (Dong et al. 2006; Dong, Morton, et al. 2012; Lemaitre et al. 1996). Alternatively, *P. chrysogenum* could activate the TOLL pathway but inhibit effector mechanisms (Behnsen et al. 2010; Chiapello et al. 2004).

3.6 Rel2-mediated defenses are inhibited by P. chrysogenum

We have shown that *P. chrysogenum* is capable of enhancing mosquito susceptibility to *P. falciparum* infection.To investigate whether *P. chrysogenum* could inhibit REL2-mediated anti-*Plasmodium* defenses, we sugar-fed conidia to genetically modified *A. stephensi* (Cp-REL2) prior to *P. falciparum* infection (Dong et al. 2011; Pike et al. 2014). These mosquitoes express REL2-s under the control of a blood meal inducible promoter that mediates transient yet potent defenses against *P. falciparum* (Dong et al. 2011; Pike et al. 2014). Cp-REL2 mosquitoes fed on *P. chrysogenum* had significantly higher ($P<0.001$) *P. falciparum* infection intensities, but no difference in prevalence, compared to sugar-fed, controls (Figure 3.3 A, Table 2). The striking increase we observed in *P. falciparum* infection of Cp-REL2 mosquitoes strongly suggests that *P. chrysogenum* inhibits REL2-mediated defense mechanisms.

We have previously shown the *A. gambiae* IMD pathway defends against *P. falciparum* ookinete and early-oocyst stages through REL2-mediated production of effector molecules

Figure 3.2 Plasmodium infection intensities after feeding on P. chrysogenum.

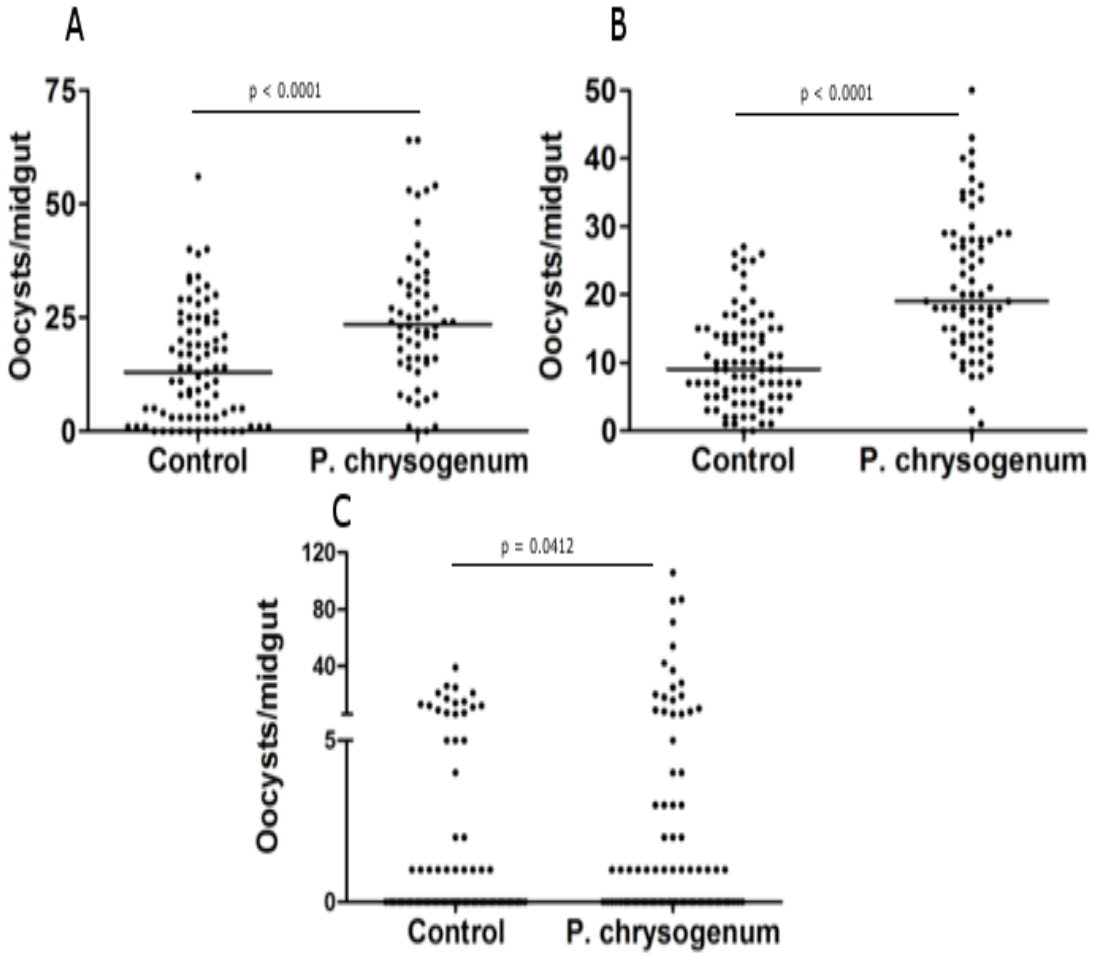

Figure 3.2: Plasmodium* infection intensities after feeding on *P. chrysogenum. *(A) P. falciparum* infection intensity ($p < 0.0001$) and prevalence ($p < 0.05$) significantly increased after feeding on *P. chrysogenum*. *(B) P. falciparum* infection intensity in antibiotic-treated mosquitoes significantly increased following feeding on *P. chrysogenum* ($p < 0.0001$). *(C) P. berghei* infection intensity ($p < 0.0412$) and prevalence ($p = 0.048$) significantly increased after feeding on *P. chrysogenum*. Three independent biological replicates were pooled for each assay. Statistical analysis of infection intensity was determined by Mann-Whitney analysis, and analysis of prevalence was determined using Fisher's Exact Test. Each circle represents a single midgut, and black horizontal bars represent the median (for *(C)* median = 0 for each).

(Garver, Dong, and Dimopoulos 2009; Garver et al. 2012; Cirimotich et al. 2010). To gain a better understanding of the infection-stage enhanced by *P. chrysogenum*, we compared ookinetes between fungi-fed and sugar-fed *A. gambiae* by 28 hours when ookinetes are still invading the midgut epithelium. The median number of ookinetes was higher in *P. chrysogenum*-fed mosquitoes compared to controls (Figure 3.3 B, Table 2), although this increase only trended towards significance (P=0.07). Despite a lack of significance, this result suggests *P. chrysogenum* could be inhibiting REL2-mediated defenses against ookinetes in the midgut lumen. Alternatively, *P. chrysogenum* could have a direct enhancing effect on parasite survival in the mosquito midgut. However, using a luciferase assay to detect viable ookinete,s we observed that *P. chrysogenum* does not directly increase ookinete development *in vitro*, supporting our hypothesis that the fungus is somehow suppressing mosquito immune defenses (Figure S2).

Aside from orchestrating defense against *P. falciparum*, the IMD pathway controls proliferation of the midgut microbiota through REL2-mediated production of antibacterial effectors (Dong, Manfredini, and Dimopoulos 2009; Garver, Dong, and Dimopoulos 2009; Meister et al. 2005). We investigated the impact of *P. chrysogenum* on the proliferation of the midgut microbiota by sugar-feeding *A. gambiae* on conidia and dissected midguts to enumerate the number of bacteria. Surprisingly, we discovered

that feeding on *P. chrysogenum* significantly increases (P<0.0001) the proliferation of the midgut microbiota (Figure 3.3 C, Table 2). This result suggests that *P. chrysogenum* strongly inhibits REL2-mediated defense against the midgut microbiota. In general, large numbers of bacteria in the mosquito midgut inversely correlates with the level of *Plasmodium* infection (Bahia et al. 2014; Dong, Manfredini, and Dimopoulos 2009). However in the context of *P. chrysogenum*, significant bacterial proliferation occurs, which is associated

Figure 3.3 Effect of P. chrysogenum on Anopheles Rel2-mediated defenses.

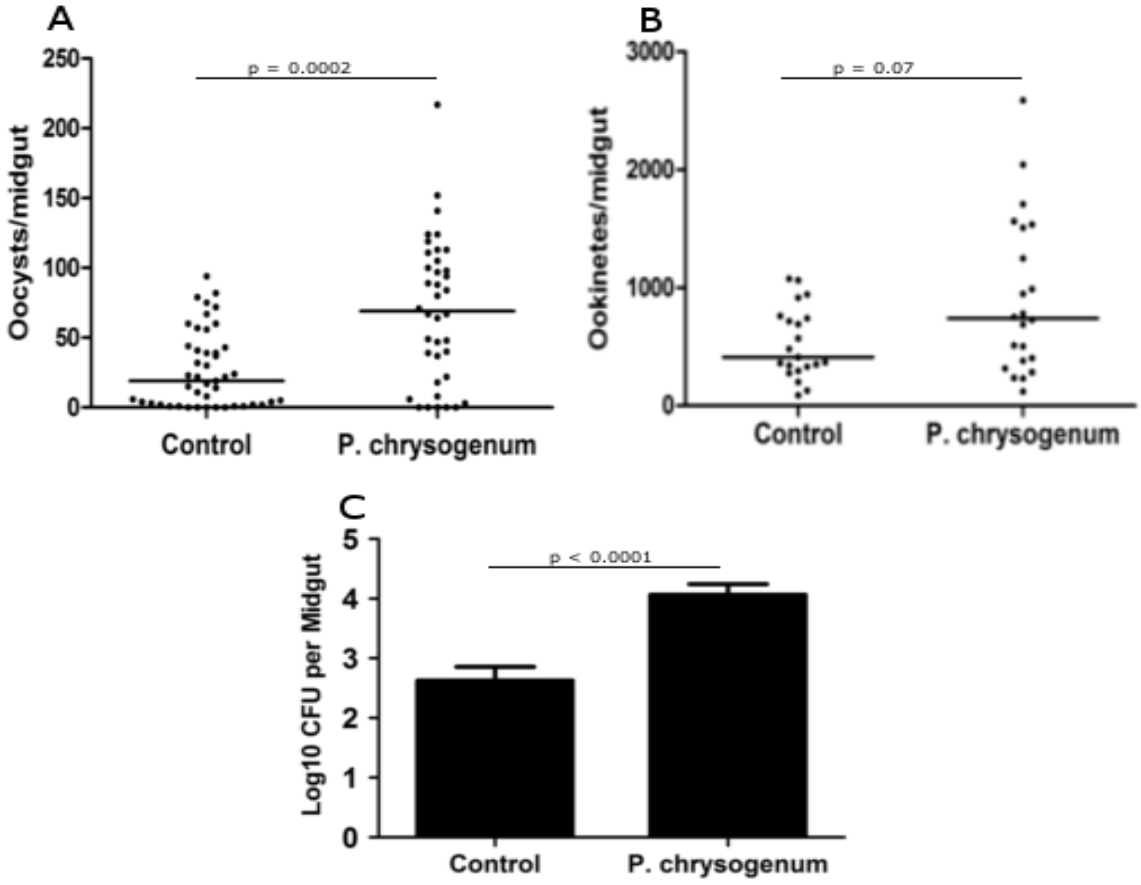

Figure 3.3: **Effect of *P. chrysogenum* on *Anopheles* Rel2-mediated defenses.** *(A) P. falciparum* infection intensity ($p < 0.0002$) significantly increased in CP-REL2 mosquitoes after feeding on *P. chrysogenum* compared to wild type *A. stephensi*. *(B) P. falciparum* ookinete intensity trended towards significance ($p = 0.07$) in *A. gambiae* fed on *P. chrysogenum*. (*C)* Midgut colony forming units (CFU) significantly increased ($p < 0.0001$) in *P. chrysogenum*-fed mosquitoes compared to controls ($p < 0.0001$). *(A)* and *(C),* three independent biological replicates were pooled for each assay, and for *(B),* two replicates. Statistical analysis of ookinete and oocyst infection intensities was determined by Mann-Whitney analysis. Analysis of midgut CFU significance was determined using an Unpaired t test. For (*A)* and *(B)*, Dots represent a single midgut and horizontal black bars represent medians. For *(C)* error bars represent standard error of the mean.

with increased *Plasmodium* infection. This implies that *P. chrysogenum* may inhibit immune pathway activation and/or effector mechanisms in the mosquito midgut. Together, these experiments strongly suggest that *P. chrysogenum* could interfere with REL2-mediated, anti-*P. falciparum* defense mechanisms in *A. gambiae*.

3.7 A P. chrysogenum-produced factor enhances mosquito susceptibility to P. falciparum infection

Fungi are capable of producing an extraordinary array of proteins and secondary metabolites, many of which have immunosuppressive qualities (Fitzpatrick, Wang, and Le 2002; Wätjen et al. 2014). To investigate if *P. chrysogenum* produces any immune modulating factors, we sugar-fed *A. gambiae* on fungal filtrate solutions removed of live fungi in conjunction with *P. falciparum* infection. Feeding on *P. chrysogenum* filtrate resulted in a significant increase in *P. falciparum* infection intensity ($P < 0.0026$), (Figure 3.4 A, Table 2). This suggests *P. chrysogenum* may produce molecular factors that enhance *P. falciparum* infection in the mosquito. We performed a crude separation of the filtrate using 3kDa filters, and the prevalence of *P. falciparum* infection increased in mosquitoes that fed on the lower molecular size fraction. This suggests a secondary metabolite or small protein could be

responsible for the increase in *P. falciparum* infection. Fungi produce many metabolites, such as mycotoxins, which are small molecules typically less than 3kDa. Alternatively, a small peptide could be responsible the enhanced infection phenotype in *A. gambiae* (Figure S3).

To investigate whether the *P. chrysogenum*-produced factor(s) was heat stable, we also fed *A. gambiae* on filtrate that was heat-inactivated at 95^{o} C for 2 hours. Surprisingly, we observed a significant increase in the intensity of *P. falciparum* infection. (Figure 3.4 A, Table T2). Mycotoxins are known for their heat-stability, and this result implicates a heat-stable molecule in mediating the observed increase in *Plasmodium* infection (Raters and Matissek 2008). In nature, mosquitoes will consume sugar and water from a variety of sources providing opportunities for fungi and/or toxins to enter the midgut environment (Müller, Xue, and Schlein 2011; Impoinvil et al. 2004). Feeding on filtrate also caused a significant increase in *P. falciparum* infection in antibiotic-treated mosquitoes (Figure 3.4 B), again suggesting the increase in infection is mediated by a bacteria-independent mechanism. We did observe a dose-dependent increase in *P. falciparum* infection using live fungi, as well as differences in the ability of *P. chrysogenum* to colonize individual mosquito midguts (Figures S4, S5). However, these filtrate experiments prove that live fungi are not required to enhance *Plasmodium* infection in the mosquito midgut.

Figure 3.4 Effect of P. chrysogenum filtrate on P. falciparum infection in A. gambiae.

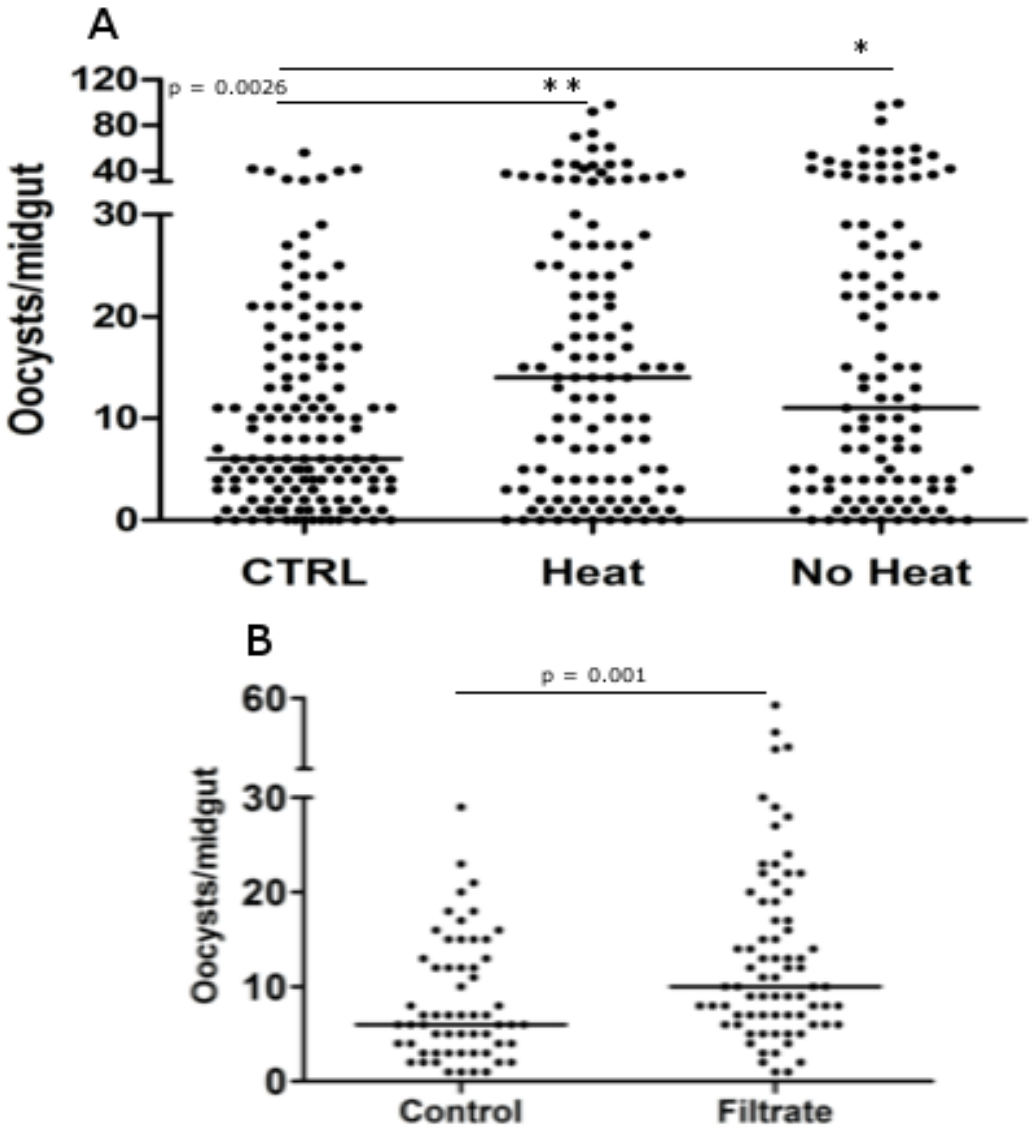

Figure 3.4: Effect of P. chrysogenum filtrate on P. falciparum* infection in *A. gambiae. *(A) P. falciparum* infection intensity increased ($p < 0.0026$) in either heat-treated (**) or un-treated (*) filtrate-fed *A. gambiae* relative to controls. *(B) Plasmodium* infection intensity increased ($p = 0.001$) in antibiotic-treated, filtrate-fed mosquitoes relative to controls. Three independent biological replicates were pooled for each assay. *(A)* statistical analysis of infection intensity was determined using a Kruskal-Wallis test followed by Dunn's post-test for multiple comparisons. *(B)* statistical analysis of infection intensity was determined using Mann-Whitney analysis. Dots represent a single midgut, and horizonal black bars represent the median infection intensity.

3.8 The JNK and JAK-STAT pathways are regulated by P. chrysogenum

We have shown that *P. chrysogenum* is capable of enhancing *Plasmodium* infection and proliferation of the midgut microbiota in *Anopheles*. To investigate our hypothesis that *P. chrysogenum* is inhibiting immune defenses against *Plasmodium* and the microbiota, we used quantitative real-time PCR (qRT-PCR) assays to assess the transcript responsiveness of immune pathways and
effector molecules in the context of *P. chrysogenum*. Blood-feeding is a profound event for the mosquito that induces complex immune gene expression patterns involved in diverse physiological processes (Dong, Manfredini, and Dimopoulos 2009). To explore if *P. chrysogenum* influences the mosquito immune response post-blood meal, we compared transcript abundance in fungi-fed mosquitoes to sugar-fed controls 24 hours after feeding on non-infectious blood (Figure 3.5 A). We observed a large increase (7-fold) in STAT, as well as a modest (2-fold) increase in REL2 and JNK transcript abundance in fungi-fed mosquitoes compared to controls (Figure). In *Drosophila*, the fat body integrates signals from the JAK-STAT and IMD pathways in response to septic injury (Agaisse et al. 2003). The increase we observed in transcript abundance of REL2 and STAT may suggest that blood-

feeding post-exposure to *P. chrysogenum* could cause septic injury to the mosquito. This idea is supported by the observed increase in JNK transcript abundance as this pathway is known to respond to oxidative stress and mediates midgut epithelial cell nitration in response to damage (Jaramillo-Gutierrez et al. 2010; Garver, de Almeida Oliveira, and Barillas-Mury 2013; Horton et al. 2011). In *Drosophila*, the JNK pathway activates the JAK-STAT pathway through cytokine signaling to stimulate intestinal cell regeneration following damage, suggesting these pathways are responsive to a *P. chrysogenum*-mediated stressor (Beebe, Lee, and Micchelli 2010). We observed a slight increase in transcript abundance (<2-fold) of TEP1, FBN9, and LRRD7, which could be attributed to activation of the IMD/REL2 axis by increased numbers of midgut bacteria as we have observed. Interestingly, there was no change in transcript abundance of REL1, suggesting the TOLL pathway is not involved in defense against *P. chrysogenum*. This observation is surprising given the emphasis placed on the TOLL pathway in anti-fungal defense. Overall, the data from this experiment suggests *P. chrysogenum* elicits a stress-response in *A. gambiae* as opposed to canonical anti-fungal defenses, and the fungus does not appear to suppress the expression of immune pathways or effectors at the transcript level.

We then investigated transcript responsiveness to *P. falciparum* invasion of the *A. gambiae* midgut epithelium in the context of *P.*

chrysogenum (Figure 3.5 B). The lack of REL1 transcript regulation confirms the TOLL pathway is not activated by *P. chrysogenum*. Again we observed an increase (2-fold) in JNK and STAT transcript abundance, confirming fungus-dependent activation of the JNK and JAK-STAT pathways. This supports our hypothesis that *P. chrysogenum* may be damaging the mosquito midgut and stimulating cell division. Alternatively, *P. chrysogenum* could be inhibiting anti-*Plasmodium* defenses, resulting in more parasites invading the midgut and a corresponding induction of the JNK and JAK-STAT pathways in response to parasite-mediated epithelial damage. Although these pathways have been implicated in anti-*Plasmodium* defense, we observed that their activation corresponds with higher *Plasmodium* infection in our model (Garver, de Almeida Oliveira, and Barillas-Mury 2013; Cirimotich et al. 2010). We previously showed that *P. falciparum* infection activates the IMD pathway, but *Plasmodium* infection in the context of *P. chrysogenum* did not result in regulation of REL2, TEP1 or FBN9 (Dong et al. 2006). Interestingly, transcript abundance of LRRD7 increased upon *Plasmodium* infection, suggesting it responds to parasite invasion of the midgut in a REL2-independent manor. Our data suggests that *P. chrysogenum* does not suppress immune defense at the transcriptional level. The enhanced *Plasmodium* infection in mosquitoes fed heat-treated filtrate led us to hypothesize that a mycotoxin may be responsible for this phenotype, as

opposed to a protein that would have been heat inactivated. This hypothesis is further supported by observed morphological changes indicative of some damage in an *Anopheles* cell line co-cultured with fungal filtrate (Figure S6). This again suggests a heat-stable mycotoxin could be responsible for the observed phenotype. We hypothesize that damage caused by this *P. chrysogenum*-associated factor primes the mosquito midgut

Figure 3.5 Transcript responsiveness to *P. chrysogenum* in blood-fed and Plasmodium-fed mosquitoes.

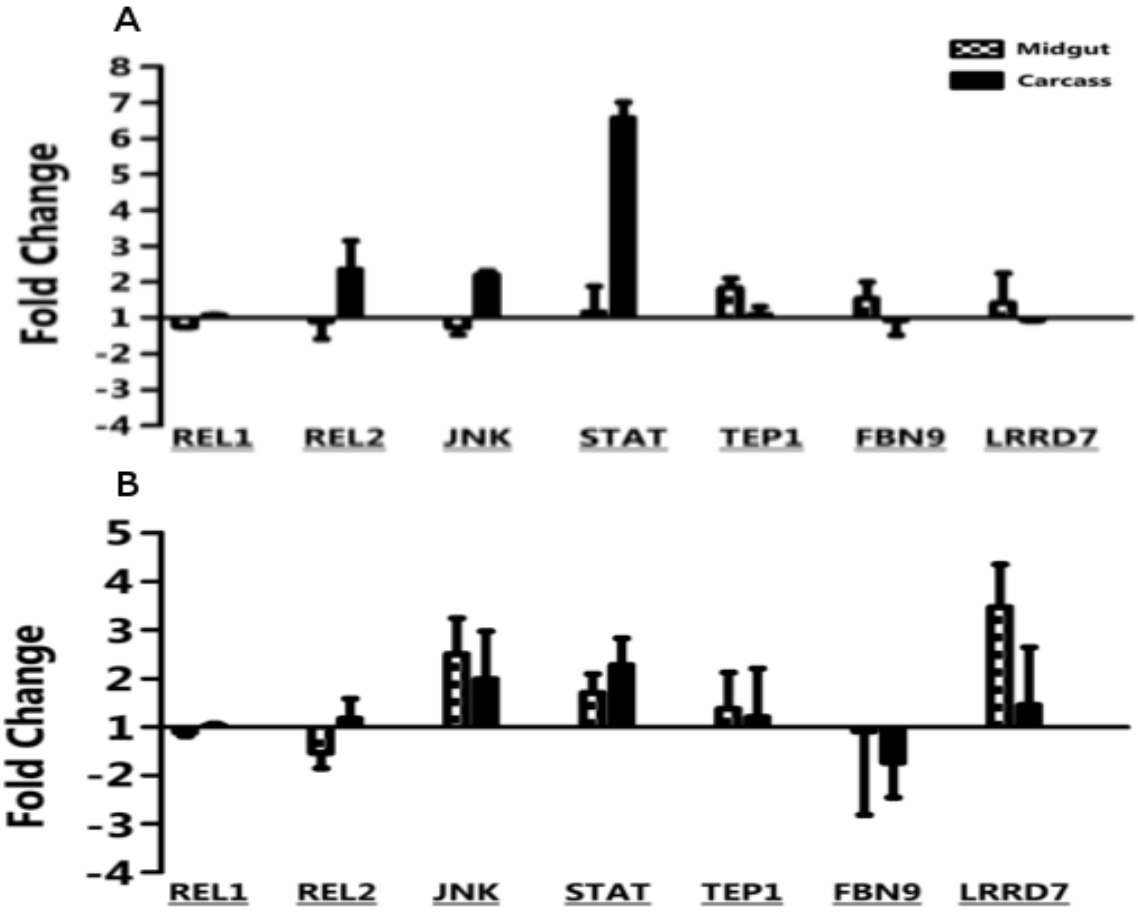

Figure 3.5: Transcript responsiveness to P. chrysogenum in blood-fed and Plasmodium-fed mosquitoes. *(A)* Fold change in transcript abundanceat 24 hours post-blood meal of *A. gambiae* immune pathways and effector molecules in blood-fed + fungus-fed. *(B)* Fold change in transcript abundance in fungus-fed *A. gambiae* 24 hours post-feeding on a blood meal of *P. falciparum* gametocytes. Data are from four biological replicates (15 tissues/replicate), and error bars represent the standard error of the mean. Hashed columns represent midguts and black columns represent carcasses. These data were processed according to Livak and Schmittgen 2001 (Livak and Schmittgen 2001).

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response to damage, and interferes with canonical anti-*Plasmodium* defense mechanisms.

3.9 P. chrysogenum does not inhibit general anti-Plasmodium defenses at the protein level

We have shown that *P. chrysogenum* induces a stress response in *A. gambiae* and that it does not suppress anti-*Plasmodium* defenses at the transcriptional level. To investigate if *P. chrysogenum* inhibits general anti-*Plasmodium* defenses at the protein level, we used a Western blot and ELISA to assess midgut epithelial cell nitration upon *P. falciparum* infection of fungi-fed mosquitoes. The JNK pathway regulates epithelial cell nitration in response to damage caused by *Plasmodium* ookinete invasion of the midgut, and we observed upregulation of the JNK pathway post-blood meal in fungi-fed mosquitoes. We hypothesized that *P. chrysogenum* could be inhibiting nitration, which would explain enhanced mosquito susceptibility to *Plasmodium* infection. However, analysis by Western blot and ELISA showed no difference in epithelial cell nitration in fungi-fed + *P. falciparum* mosquitoes compared to controls (Figure 3.6 A, B, C). Nitration was actually higher in fungi-fed + *P. falciparum* mosquitoes compared to controls, which correlates well with our data showing an increase in invading ookinetes and higher *Plasmodium* infection intensity. This suggests that the process of epithelial cell nitration is unaffected by *P. chrysogenum*.

Nitration modifies ookinetes making them visible to the mosquito complement system, which in turn lyses parasites through a TEP1-dependent mechanism (G. de A. Oliveira, Lieberman, and Barillas-Mury 2012). TEP1 plays a critical role in anti-pathogen defense against bacteria, fungi, and *Plasmodium* (Stephanie Blandin et al. 2004; Gubb et al. 2010; Yassine, Kamareddine, and Osta 2012; Garver et al. 2012; Bou Aoun et al. 2011). Since the nitration system is unaffected by *P. chrysogenum*, we investigated if *P. chrysogenum* suppresses TEP1 protein levels in the mosquito. A relative of *P. chrysogenum* produces an alkaline protease that inactivates the orthologue of TEP1 in mammals, and through homology we identified a similar protease in the genome of *P. chrysogenum* (Behnsen et al. 2010). We hypothesized that fungal-mediated degradation of TEP1 could explain the observed increase in *Plasmodium* susceptibility. However, sugar-feeding on *P. chrysogenum* had no effect on the amount of TEP1 protein in the *A. gambiae* hemolymph (Figure 3.6 D). This suggests that the observed increase in *A. gambiae* susceptiblity to *Plasmodium* infection is not a result of TEP1 degradation by *P. chrysogenum*. Since pathogen lysis by the mosquito complement system is a multi-step process involving many proteins, it is possible that *P. chrysogenum* inhibits another step in this process (Povelones et al. 2013). These results demonstrate that *P. chrysogenum* does not inhibit major anti-pathogen effector mechanisms at the protein level. *P.*

chrysogenum or an associated-factor may damage the midgut and elicit a stress response that impairs anti-*Plasmodium* defense through an atypical and uncharacterized mechanism.

Figure 3.6 The effect of P. chrysogenum on general anti-Plasmodium defense in A. gambiae.

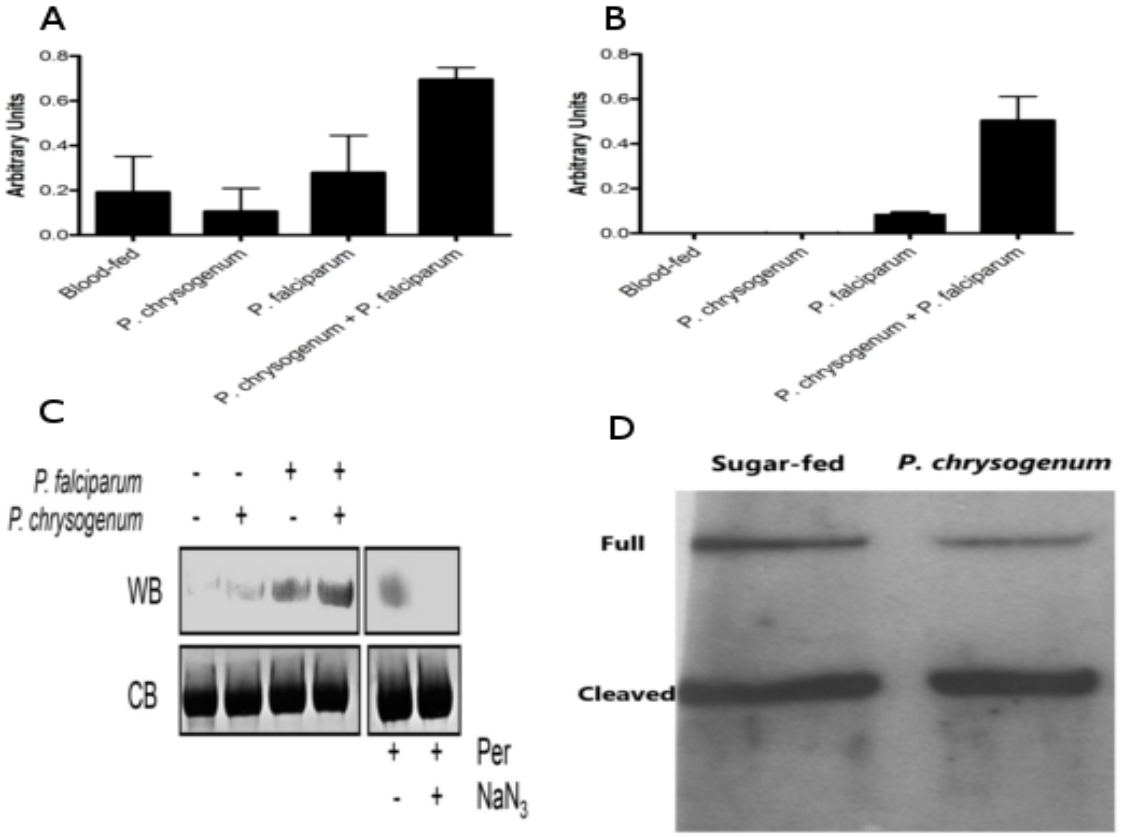


Figure 3.6: **The effect of *P. chrysogenum* on general anti-*Plasmodium* defense in *A. gambiae*.** *(A)* Densitometric analysis of nitration in the midgut epithelium of *A. gambiae* following different treatments showed no statistical difference ($p = 0.10$) between the groups. *(B)* Same as *(A)* except blood-fed arbitrary units subtracted from all groups to remove background nitration during blood-feeding. Subtracting background units resulted in significant differences ($p = 0.008$) between the treatments, and specifically between ($p < 0.05$) mosquitoes fed *P. chrysogenum* + *P. falciparum* compared to *P. falciparum* alone. *(C)* Western blot of nitration increased in *P. falciparum* + *P. chrysogenum* mosquitoes. *(D)* Western blot showing no difference in TEP1 protein abundance in the *A. gambiae* hemolymph after feeding on *P. chrysogenum*. *(A)* and *(B)*, samples represent three biological replicates. Statistical significance was calculated by One-way ANOVA followed by Tukey's Multiple Comparison Test for between group comparisons. Arbitrary units represents fluorescence. *(C)* WB is nitration and CB is the loading control. Per is the horseradish peroxidase positive control for nitration and NaN_3 is used to block nitration as a negative control. Analyzed similar to (G. de A. Oliveira, Lieberman, and Barillas-Mury 2012) *(D)* Full length versus cleaved TEP1 protein from pools of mosquito hemolymph.

3.10 Discussion

The *Anopheles* mosquito's endogenous microbiota are capable of stimulating immune responses that coordinate anti-*Plasmodium* defenses in the midgut and restrict parasite infection (Dong, Manfredini, and Dimopoulos 2009; Bahia et al. 2014). While bacteria have been shown to mediate anti-*Plasmodium* defense through activation of the IMD pathway, we show here that the common ascomycete fungus, *P. chrysogenum*, can increase *Anopheles* susceptibility to *Plasmodium* infection. To our knowledge, this is the first report characterizing tripartite interactions in the midgut between the *Anopheles* immune system, *Plasmodium*, and a non-entomopathogenic fungus. *P. chrysogenum* activates immune pathways previously implicated in anti-*Plasmodium* defense, and it also does not inhibit general or parasite-species-specific immune responses (Cirimotich et al. 2010; Dong et al. 2006; Bahia et al. 2011; Garver, de Almeida Oliveira, and Barillas-Mury 2013). Indeed, fungal-mediated enhancement of *Plasmodium* infection in the mosquito is intriguing because some known defense mechanisms are not disrupted. Our results strongly suggest *P. chrysogenum* is responsible for an atypical form of immune suppression. We believe the increase in *Plasmodium* infection is due to fungal priming of the midgut environment, possibly mediated by damage, which predisposes the mosquito immune system to

respond primarily to stress caused by ookinete invasion of the midgut epithelium. This modified immune response is unlike the archetypical anti-*Plasmodium* response involving the production of anti-pathogen effector molecules. The enhancement of *Plasmodium* infection in mosquitoes fed on heat-inactivated fungal filtrate, strongly suggests that a mycotoxin may be responsible for the observed phenotype. These findings may have significant implications in regards to the epidemiology of naturally occurring *Plasmodium* infection in *Anopheles* mosquitoes. Like many species of *Penicillium* fungi, *P. chrysogenum* is non-pathogenic to mosquitoes and may not exert strong selective pressure for the development of mosquito resistance. *Penicillium* fungi could also be naturally associated with *Anopheles* mosquitoes, and our work suggests that mosquitoes can acquire fungi through sugar-feeding. The plausibility that an environmentally stable mycotoxin can enhance *Plasmodium* infection, suggests live fungi are not required for the modification of mosquito immune responses. Therefore, it could be possible that a single sugar or water feed in nature can influence the course of pathogen infection in the mosquito. It may be prudent to investigate the midguts of field-caught mosquitoes from regions experiencing epidemic malaria to investigate any correlation with *Plasmodium* infection. Although *P. chrysogenum* or its associated factor would not be useful for interrupting *Plasmodium* transmission, identification of the factor enhancing *Plasmodium*

infection could be used to increase parasite infections in a research or commercial setting. In summary, we have discovered that *P. chrysogenum* is capable of enhancing *Plasmodium* infection in *A. gambiae*, and our findings warrant further investigations into interactions between mosquito vectors, human pathogens, and the mosquito mycobiome.

3.11 Supplementary Figures and Tables

S1 P. chrysogenum culture and fungal growth in the mosquito crop

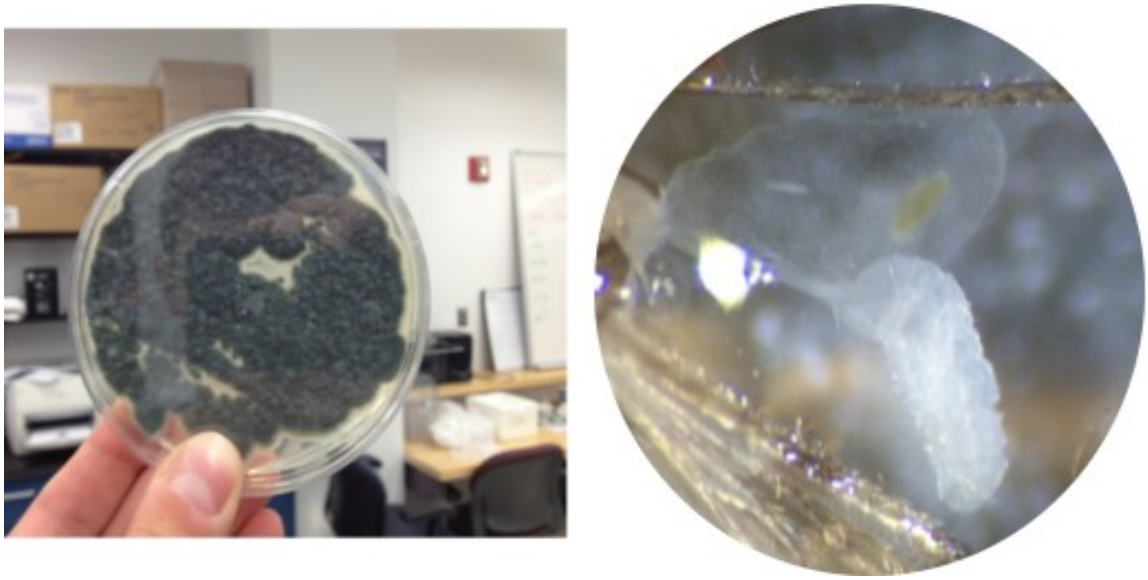


Figure S1: P. chrysogenum culture and fungal growth in the mosquito crop. Left, a SDA plate culture of *P. chrysogenum* forming lawn of conidia after 1-2 weeks incubation. Right, Mass of *P. chrysogenum* growing in the crop of an adult female *A. gambiae* 4 days post-feeding on a fungi-laced sugar meal.

S2 Effect of live *P. chrysogenum* or filtrates on *P. berghei* ookinete viability in vitro.

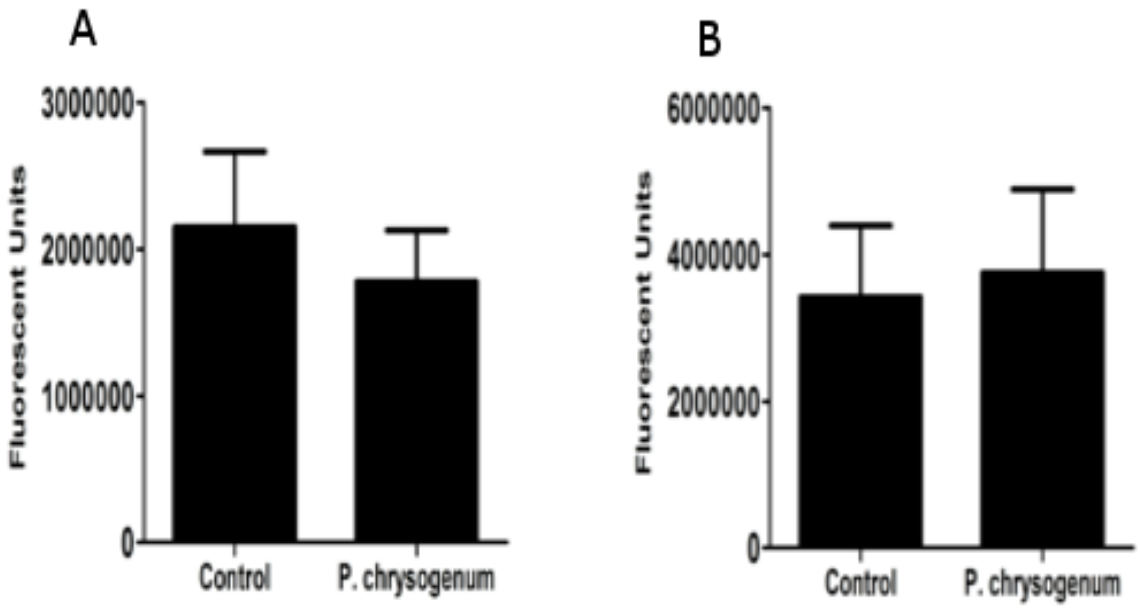
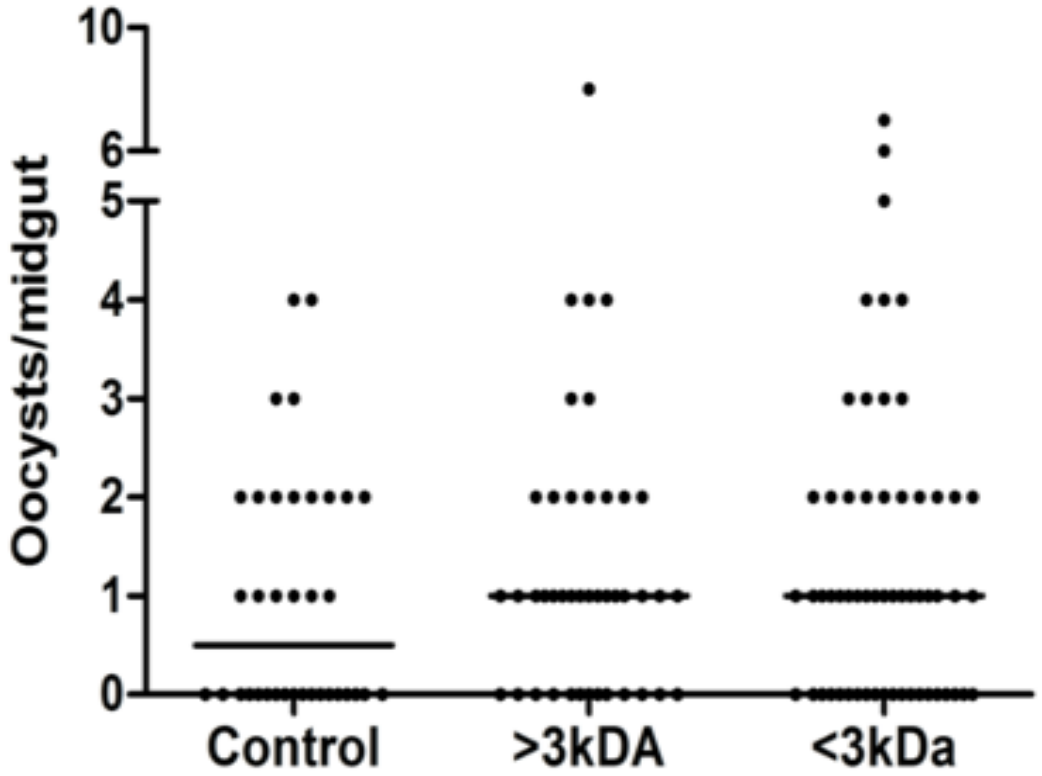


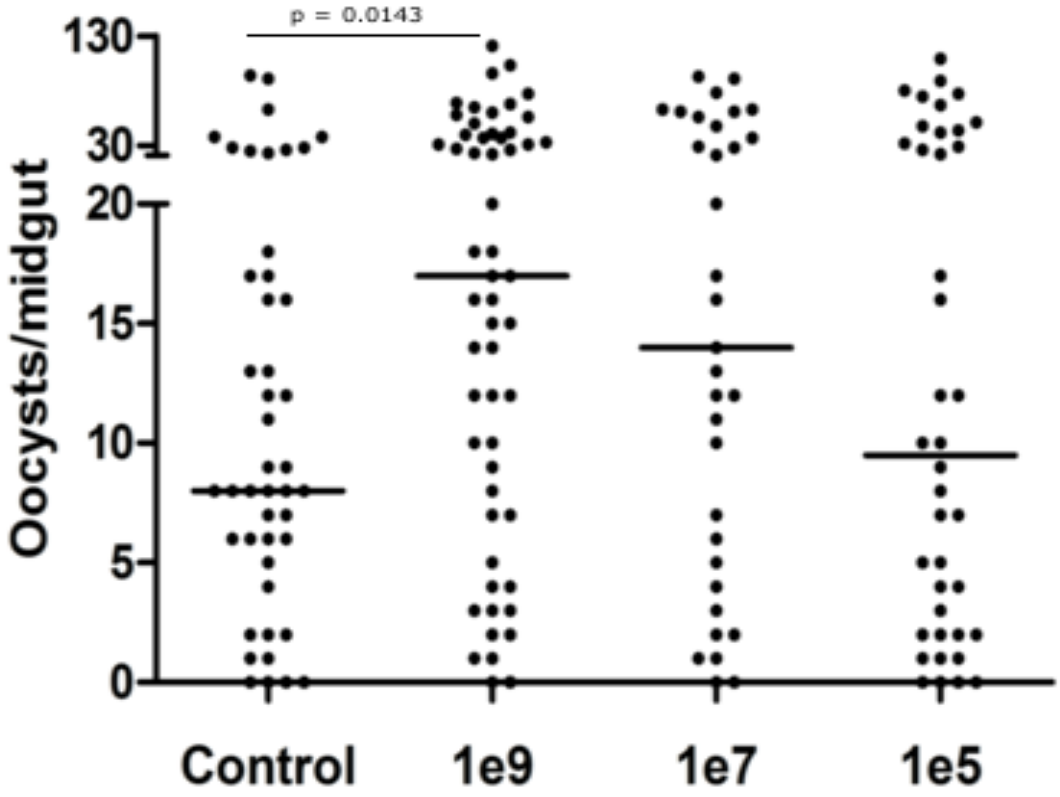
Figure S2: Effect of live P. chrysogenum or filtrates on P. berghei ookinete viability in vitro. *(A)* No difference in ookinete viability between untreated controls and live fungi-treated experimental group. *(B)* No difference in ookinete viability between untreated controls and filtrate-treated experimental group. Both *(A)* and *(B)* assays contain three biological replicates, and statistical significance was determined using a Mann-Whitney test. Error bars represent standard error of the mean, and fluorescent units refer to the mean arbitrary fluorescence of each sample pool.

S3 Effect of filtrate fractionation above and below 3kDa on *P. falciparum* infection in *A. gambiae*.



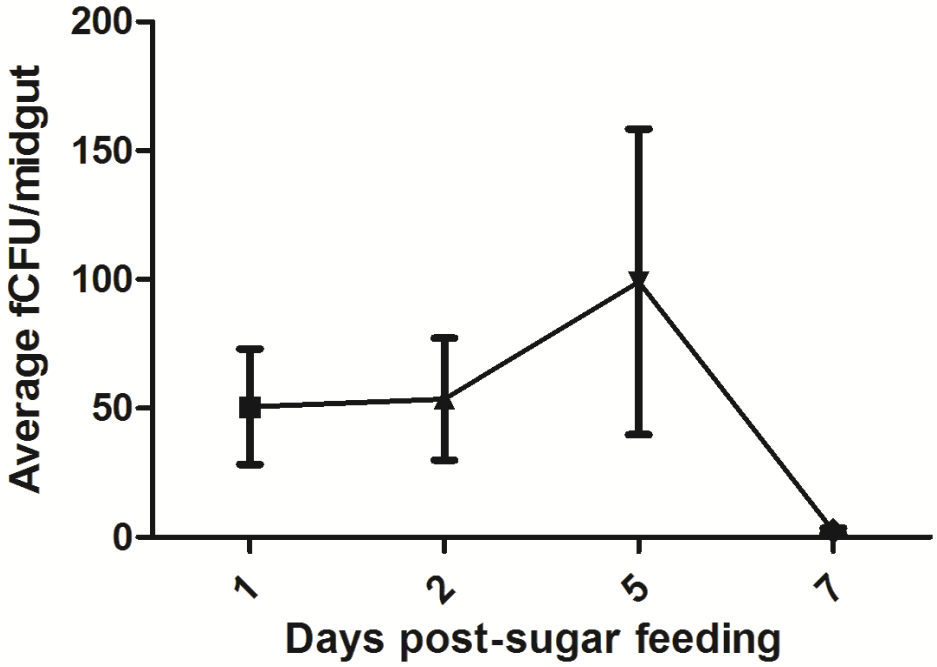
S3: Effect of filtrate fractionation above and below 3kDa on P. falciparum infection in A. gambiae. The prevalence of *P. falciparum* infection increased significantly ($p = 0.007$) in mosquitoes fed on fractionated filtrate below 3kDa. Data are from two biological replicates, black horizontal bars represent the median oocysts per midgut, each point represents the number of oocysts in a single midgut. Statistical significance of infection intensity was calculated by Kruskal-Wallis test followed by Dunn's post-test for multiple comparisons. Analysis of prevalence was performed using Fisher's Exact Test.

S4 Effect of varying *P. chrysogenum* dosage on Plasmodium infection in *A. gambiae*.



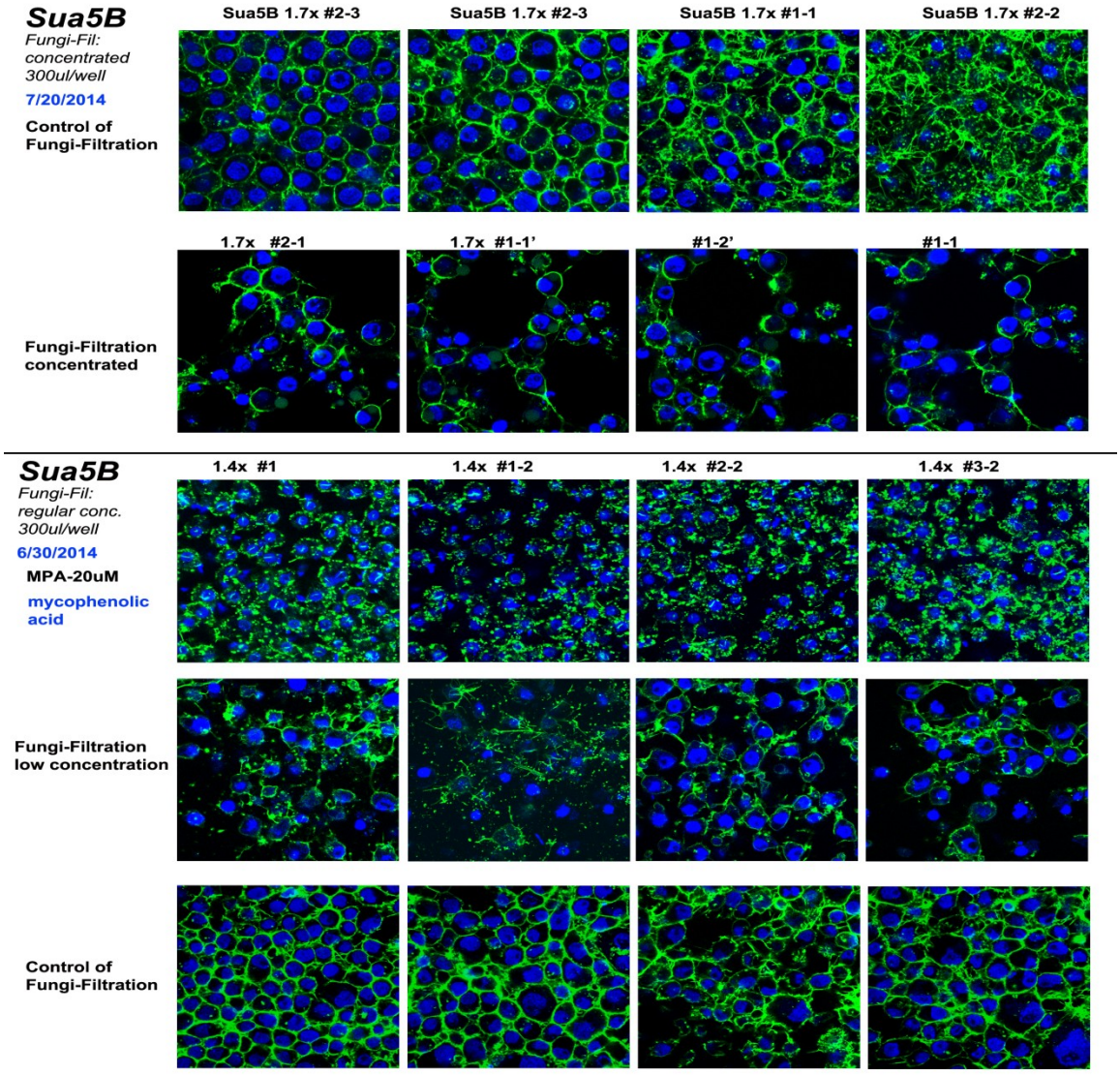
S4: Effect of varying P. chrysogenum dosage on Plasmodium infection in A. gambiae. *Plasmodium* infection intensities varying between different dosages of *P. chrysogenum*. Although the black horizontal bars representing median infection intensity demonstrates a dose-dependent effect on the observed enhances infection, only the 1e9 group was significantly ($p = 0.0143$) higher than the control. Two biological replicates were pooled at each dosage and each dot represents a single midgut. Statistical significance was calculated by using a Mann-Whitney test followed by Bonferonni's correction for multiple comparisons.

S5 Fungal colony forming units recovered from *A. gambiae* midguts over the course of 7 days post-fungal feeding.



S5: S5 Fungal colony forming units recovered from A. gambiae midguts over the course of 7 days post-fungal feeding. Midguts were dissected from fungi-fed mosquitoes at days 1, 2, 5 and 7 post-feeding to determine if fungi were actively growing within the mosquito. It appears that by day 5, a few mosquitoes are capable of supporting large amounts of fungal growth effectively pulling the mean higher. However, by day 7 most mosquitoes do not have cultivable fungi in their midguts. Three biological replicates were performed at each time point, vertical lines represent standard error of the mean and each point on the line represents the mean fungal CFUs per midgut.

S6 Effect of filtrate on *A. gambiae Sua5b* cell line viability in vitro.



S6: Effect of filtrate on A. gambiae Sua5b cell line viability in vitro. Top, effect of concentrated fungal filtrate on Sua5b cells compared to untreated controls. Bottom, comparison of cells incubated with mycophenolic acid or unconcentrated fungal filtrate relative to controls. Sua5b cells incubated with filtrate displayed an intermediate phenotype compared to untreated controls and MPA-treated positive controls. Green = phalloidin staining of F-actin, and Blue = DAPI staining of cell nuclei.

3.1 Primers Used

Gene Target	Primer Name	Primer Sequence	Primer Use
S7	S7-Forward	CCATCCTGGAGGATCTGGTA	qRT-PCR
	S7-Reverse	GATGGTGGTCTGCTGGTTCT	
Tep1	TEP1-Forward	TCCAGCGTATGTGGTTGTGT	qRT-PCR
	TEP1-Reverse	TCGCACAAATTCTGCTTGTC	
Fbn9	FBN9-Forward	TTGTGATGAAGGAGCACAGC	qRT-PCR
	FBN9-Reverse	GCTTGATCCAACCGACTGAT	
Lrrd7	LRRD7-Forward	TCGGTGAGCAACAGTTTGAC	qRT-PCR
	LRRD7-Reverse	CAGGTCGAGATGGGTGAACT	
REL1	REL1-F	TAGCCCGTAAGCATCCATTC	qRT-PCR
	REL1-R	TGCCAATGGTCTGTTGGTAA	
REL2	REL2-F	CGGAGAAGTCGAAGAAAACG	qRT-PCR
	REL2-R	GTTGCGGATCCACCTGATAG	
JNK	JNK-F	TGCCAGGTCATACAGATGGA	qRT-PCR
	JNK-R	CCCAAAGTCGAGGATTTTCA	
STAT-A	STAT-F	TACAACGAAACGACCAAGCA	qRT-PCR
	STAT-R	GGTCCATACCGAAAAGACGA	
ITS	ITS5-F	TCCTCCGCTTATTGATATGC	Sequencing
	ITS4-R	GGAAGTAAAAGTCGTAACAAG G	

3.2 Summary Statistics from Selected Experiments

Figure 3.2A	**Control**	***P. chrysogenum***
N	83	58
Mean	14	25
Median	13	23.5
Prevalence	88%	97%
% Change (Median)		+80%
Figure 3.2B	**Control**	***P. chrysogenum***
N	87	72
Mean	10	21
Median	9	19
Prevalence	98%	99%
% Change (Median)		+111%
Figure 3.2C	**Control**	***P. berghei***
N	104	89
Mean	2.8	7.9
Median	0	0
Prevalence	30%	48%
% Change (Median)		+80%
Figure 3.3A	**Control**	***CP-REL2***
N	45	38
Mean	27	69
Median	19	69
Prevalence	89%	87%
% Change (Median)		+363%
Figure 3.3B	**Control**	***P. chrysogenum***
N	21	22
Mean	529	912
Median	412	740
Prevalence	100%	100%

Figure 3.4A	***Control***	***Heat***	***No Heat***
N	143	120	109
Mean	10	18	20
Median	6	14	11

Prevalence	90%	92%	91%	
% Change (Median)		+2%	1%	
Figure 3.4B	**Control**	***P. chrysogenum***		
N	59	75		
Mean	8.5	13.5		
Median	6	10		
Prevalence	100%	100%		
Figure S3	**Control**	***<3kdu***	**3>kdu**	
N	36	58	42	
Mean	1	1.33	1.38	
Median	0.5	1	1	
Prevalence	50%	78%	52%	
% Change (Median)		+50%	50%	
Figure S4	***Control***	***10^9***	***10^7***	***10^5***
N	45	55	33	40
Mean	16	27.4	26.8	23.3
Median	8	17	14	9.5
Figure S5	**DAY 1**	**DAY 2**	**DAY 5**	**DAY 7**
N	40	39	40	40
Mean	51	54	99	2.4
Median	6	2	2	0

Conclusion and Discussion

In the preceding work, we sought to characterize bacteria-independent immune defense and those defenses mediated by fungi, two relatively

unexplored areas in *Anopheles* immunity. Using various molecular tools in combination with *Plasmodium* infection assays, we have successfully identified the existence of bacteria-independent anti-*Plasmodium* defenses and characterized an enhanced infection phenotype mediated by a non-pathogenic fungus.

In Chapter 2, we used whole genome microarray analysis to characterize the *A. gambiae* bacteria-independent, anti-*P. falciparum* transcriptome, which lead to the identification of the existence of bacteria- and IMD pathway-independent anti-*Plasmodium* defenses. These findings add additional complexity to our understanding of mosquito anti-*Plasmodium* defenses in the mosquito, and additional targets to exploit in order to disrupt parasite transmission. Many questions remain; What specific immune processes are regulated by SRPN7 and CLIPC2? Which pathways do these effectors belong too? What transcription factor mediates their production? Is their function dependent on the genetic background of the mosquito? Are there upstream pattern recognition receptors mediating their infection responsiveness? Aside from these remaining questions, future directions should include a rigorous biochemical characterization of SRPN7 and CLIPC2. There are also other regulated gene transcripts in the bacteria-independent transcriptome awaiting characterization. These studies could provide many additional targets for both gene function studies and

translational applications utilizing transgenic mosquitoes specifically refractory to *Plasmodium* infection.

In Chapter 3, we confirmed our hypothesis that fungi could modulate immune defense mechanisms in the mosquito. However, we found that *P. chrysogenum* introduced by way of a sugar meal actually enhances malaria infection in *Anopheles* mosquitoes. In depth examination showed that *P. chrysogenum* can inhibit anti-bacterial and anti-*Plasmodium* defenses in mosquitoes, possibly through a novel mechanism since some of the principle defense effector molecules were not affected at the transcript or protein level. We also discovered that the fungus can produce a heat-stable factor that is capable of mediating increased malaria infection. Discovery of this molecule could lead future studies to identify the mechanism and help dissect immune the immune response involved in the enhanced infection phenotype. Furthermore, this molecule could be used in a laboratory or commercial setting to increase infection in mosquitoes used in the production of sporozoite-based vaccines or other experiments. This work also serves as justification for further characterization of mosquito-fungi interactions and if they have a meaningful impact on malaria transmission.

In summary, this work serves as the starting point for further exploration of the *Anopheles* immune system in regards to pathogen defense mechanisms and host-microbiome interactions.

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Curriculum Vitae

Education

2009-Present: Johns Hopkins Bloomberg School of Public Health, Baltimore, MD Ph.D. in Molecular Microbiology and Immunology (expected thesis defense in August 2014)

2002-2006: University of Vermont, Burlington, VT B.S. in Biology

Research Positions

2009-Present: Doctoral research in the laboratory of Dr. George Dimopoulos. Department of Molecular Microbiology and Immunology, Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health. Identified the existence of bacteria-independent anti-*Plasmodium* immune defenses in *Anopheles gambiae*. Isolated fungi from field-caught mosquitoes and discovered the ability of *Penicillium* mold to modulate *Plasmodium* susceptibility in *Anopheline* mosquitoes. Collaborated with two postdoctoral colleagues on *Anopheline* microbiome and immune defense projects that have been accepted and submitted for publication, respectively. Designed a project for a master's student to explore tripartite interactions between a *Candida*

yeast species, *Plasmodium falciparum*, and the *Anopheline* innate immune system.

2007-2009: Research Fellow in the laboratory of Dr. Jerry Weir. Division of Viral Products, Center for Biologics Evaluation and Research (CBER), U.S. Food and Drug Administration. Tested all U.S. manufactured seasonal influenza vaccines to confirm identity to circulating influenza viruses prior to approval for public use. Developed cell culture-based protocols for rapid scale-up of influenza vaccine potency reagent production and deployment in the event of a global avian influenza pandemic.

2006-2007: Research Assistant in the laboratory of Dr. Brian Beckage. Department of Plant Biology, University of Vermont. Collected vegetation data from field sites in Vermont and the Everglades, FL to assess the impact of invasive species and global climate change on plant composition and fire regime.

Awards and Fellowships

2013: EMBO F500 Travel Award, EMBO Molecular and Population Biology of Mosquitoes and Other Disease Vectors: From Basic Vector Biology to Disease Control, Kolymbari, Greece 15-19 July 2013.

2010: The Dr. Lloyd and Mae Rozeboom Scholarship
Award for the study of Medical Entomology

2007-2009: Oak Ridge Institutes for Science and Education Fellowship
U.S. Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER)

Publications

Sandiford, S. L., Y. Dong, A. Pike, **B. Blumberg**, A.C. Bahia, and G. Dimopoulos. (In submission). *Cytoplasmic actin is an extracellular insect immune factor which is secreted upon immune challenge and mediates phagocytosis and direct killing of bacteria, and is a Plasmodium antagonist*. *PLoS Pathogens*

Bahia, A. C., Y. Dong, **B. Blumberg**, G. Mlambo, A. Tripathi, O.J. Benmarzouk-Hidalgo, O. J., R. Chandra and G. Dimopoulos. 2014. *Exploring Anopheles gut bacteria for Plasmodium blocking activity*. *Environ Microbiology* doi: 10.1111/1462-2920.12381.

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Schmeisser, F., J.E. Adamo, **B. Blumberg**, R. Friedman, J. Muller, J. Soto and J.P. Weir. 2012. Production and characterization of mammalian virus-like particles from modified vaccinia Ankara vectors expressing influenza H5N1 hemagglutinin and neuraminidase. *Vaccine* 14;30(23):3413-22

Martinsen, E.M., **B. J. Blumberg**, R. J. Eisen and J. J. Schall. 2008. Avian haemosporidian parasites (phylum Apicomplexa) from northern California oak woodland and chaparral habitats. *Journal of Wildlife Diseases* 44:260-268.

Oral Presentations

Blumberg, B.J. "Fungi modulate *Anopheline* susceptibility to *Plasmodium falciparum* infection," presented in session on Symbionts, Microbiota & Immunity at the EMBO Molecular and Population Biology of Mosquitoes and

Other Disease Vectors: From Basic Vector Biology to Disease Control, Kolymbari, Greece 15-19 July 2013.

Blumberg, B.J. "Bacteria-independent anti-*Plasmodium* immune defense activation," presented during young researcher Nature Immunology Prize competition for best presentation at Training the Innate Immunity: Immunological Memory in Innate Host Defense, Nijmegen, Netherlands 28-29 July 2012.

Blumberg, B.J. "The design and use of Modified Vaccinia Ankara (MVA) virus recombinants as a mammalian influenza HA expression system," presented at the NIH post-baccalaureate seminar series, Bethesda, MD, 30 April 2008.

Poster Presentations

Blumberg, B.J., G. Barringer III, A. Pike, A. Clayton and G. Dimopoulos. "The *Anopheline* anti-fungal defense system" poster presented at Research Advances in Malaria: Malaria Transmission from the mosquito midgut to the mammalian liver, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, 24-25 April 2013.

Blumberg, B.J., S. Trop, S. Das and G. Dimopoulos. "Bacteria-independent anti-*Plasmodium* immune defense activation" poster presented as part of the 2012 Summer Frontiers Symposium on "Training the innate immunity: immunological memory in innate host defense," Nijmegen, The Netherlands, 28-29 June 2012.

Blumberg, B.J., S. Trop, S. Das and G. Dimopoulos. "Bacteria-independent anti-*Plasmodium* immune defense activation" poster presented at the 2011 American Society of Tropical Medicine and Hygiene Annual Meeting, Philadelphia, PA, 4-8 December 2011.

Blumberg, B. J., F. Schmeisser, and J. P. Weir. The Design and Use of Modified Vaccinia Ankara (MVA) Virus Recombinants as a Mammalian Influenza HA Expression System. NIH Spring Research Festival, Bethesda, MD. 7 May 2008. Bethesda, Maryland.

Teaching Experience

2013: Teaching Assistant for Malariology

Managed the online documents associated with the course. Provided mentoring to students on an ad-hoc basis.

2013: Teaching Assistant for Introduction to Biomedical Sciences

Constructed and presented a lecture on the endocrine system. Designed formative and summative assessments on the endocrine system. Lead students through case-studies to reinforce learned biomedical concepts.

2012: Teaching Assistant for The Biology of Parasitism

Guided students in identifying parasites by light microscopy and understanding diverse parasite life cycles. Coordinated and maintained a live experimental *Plasmodium* and schistosome co-infection of mice for student data collection throughout the course. Proctored laboratory and in-class summative assessments of learned material.

Mentoring Experience

2013: Mentor to a master's student in the laboratory of Dr. George Dimopoulos. Trained an incoming master's student in molecular and microbiological techniques required for the study of mosquito-yeast interactions. Assisted the student in data analysis, project troubleshooting, and experimental design.

2012: Mentor to an undergraduate student in the laboratory of Dr. George Dimopoulos. Trained a visiting summer student in RNAi methodology used to characterize candidate anti-*Plasmodium* defense genes in the mosquito *Anopheles gambiae*.

Extracurricular

2013-2014: Contributor to Public Health United Podcast (http://www.publichealthunited.org/). Participated in a panel discussion tracing science in the news to its peer-reviewed source to emphasize the importance of effective and truthful science communication to the general public. Wrote an article citing the importance of using inclusive language when communicating science to the general public in order to avoid alienating those seeking to be informed in matters of public health importance.